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(54) Title: PEPTIDYL COMPOUNDS COMPRISING AN ITIM MOTIF WHICH REGULATE HEMATOPOIETIC FUNCTION AND THEIR USE

(57) Abstract

The present invention is directed to a novel product and method for regulating hematopoietic cell function and a novel product and method for identifying compounds capable of regulating inflammation. The present invention includes a method to regulate hematopoietic cell function by contacting a cell with a regulatory reagent capable of altering the activity of a molecule including PTP1C, PTP1D, ITIM-p160 and ITIM-p70. The present invention also relates regulatory reagents capable of regulating the activity of PTP1C, PTP1D, ITIM-p160 and ITIM-p70, including nucleic acid molecules having sequences that encode such reagents and antibodies raised against such reagents. The present invention also includes a therapeutic composition comprising such reagents and their use to protect animals from inflammation.

p58.183.1	vnredsdeqdpqeVtYaqLnhcvftqrkit--	288-317
p58.183.2	rpsqrpktpptdiIvYteLpnaep--------	318-341
p58.EB6.1	ansedsdeqdpqeVtYtqLnhcvftqrkit--	287-316
p58.EB6.2	rpsqrpktpptdiIvYteLpnaesrskvvscp	317-346
p70.1	ansedsdeqdpeeVtYaqLdhcvftqrkit--	383-412
p70.2	rpsqrpktpptdtIlYteLpnakprskvvs--	413-442
Ly49A	--------mseqeVtYsmVrfhksaglqkq--	1-22
Ly49C	--------mseqeVtYttLrfhkssglqnp--	1-22
Ly49G4	--------mseqeVtYstVrfhessrlqkl--	1-22
NKG2A.1	--------mdnqgViYsdLnlppnpkrqqr--	1-22
NKG2A.2	kgnkssilateqeItYaeLnlqkasqdfqg--	25-54
hFcgRIIB1	npdeadkvgaentItYslLmhpdaleepdd--	259-288
mFcgRIIB1	dleeaapktaentItYslLkhpealdeete--	294-323
hCD22.2	hslgcynpmmedgIsYttLrfpemn-iprtg-	747-771
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mCD22.6	qfgagkrpqakedVdYvtLkh----------	842-862
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PEPTIDYL COMPOUNDS COMPRISING AN ITIM MOTIF WHICH REGULATE HEMATOPOIETIC FUNCTION AND THEIR USE

This invention was made in part with government support under AI-21768, AI-20519 and DK-47121, all awarded by the National Institutes of Health. The government has certain rights to this invention.

Cross Reference To Related Applications

The present application is a continuation-in-part of U.S.S.N 08/397,628, filed on February 28, 1995 which is incorporated herein in its entirety by this reference.

Field Of The Invention

The present invention relates to a product and process for regulating signal transduction pathways. In particular, the invention provides for peptides and mimetics which regulate the responses of hematopoetic cells. As such, the present invention provides a product and process which regulates inflammatory and immune responses and further provides for the development of drug screening assays.

Background Of The Invention

In all multicellular organisms, cell to cell communication coordinates the growth, differentiation and metabolism of the multitude of cell types contained in an organism. Communication between cells over long distances is facilitated by extracellular products (ligands) which act as signal molecules. Signal molecules travel through the organism to specific cells, where the signals induce a specific response only on those target cells that have receptors capable of binding those signal molecules. Binding of a signal molecule to a receptor initiates complex intracellular reactions within the cell bearing the receptor, triggering modifications of molecules present in the cell or altering patterns of gene expression, resulting in alteration of biological functions of the cell. This process is called signal transduction.

Signal transduction pathways are used by cells to enable a cell to adjust to its environment. A signal transduction pathway transmits signals received outside of a cell, across the cell's plasma membrane, through the cytoplasm of the cell and into the nucleus. Signals transmitted in this manner typically result in alteration of gene expression in the cell, though non-transcriptional events, such as certain changes in membrane structure and permeability, can also be regulated by intracellular signaling effected by the subject compositions. Multiple cellular elements can be responsible for regulation of signal transduction in a cell. Such elements can include initiator molecules, transducer molecules,

amplifier molecules, target molecules and second messenger molecules. As used herein, "molecule" includes proteins, lipids and ions (such as Ca^{2+}).

Initiator molecules are capable of initiating signal transduction in a cell. An example of an initiator molecule is an extracellular ligand capable of binding to a transducer molecule comprising a membrane-bound receptor on the surface of a cell. Extracellular ligands can include, for example hormones, growth factors, antigens, differentiation agents, antibody molecules, histocompatibility molecules, mitogens, and other soluble or cell bound molecules. Following binding to an initiator molecule, a receptor/transducer molecule transmits a signal across the plasma membrane of a cell to intracellular amplifier molecules. Transducer molecules can include any cell surface receptor having cytoplasmic regions capable of interacting with intracellular proteins involved in signal transduction. Transducer molecules can include a catalytic domain or can recruit catalyticly active molecules to the receptor site, such as kinases or phosphatases. Some examples of transducer molecules include, Fc receptors, T cell antigen receptors, B cell antigen receptors, major histocompatibility molecule (MHC) receptors, tyrosine kinase receptors, alpha and beta adrenergics, or other G protein-linked receptors, as well as cytokine receptors, growth factor receptors, CD22, CD19 and many others.

Receptors can be comprised of multiple proteins referred to as subunits, one category of which is referred to as a multisubunit receptor is a multisubunit immune recognition receptor (MIRR). MIRRs include receptors having multiple noncovalently associated subunits and are capable of interacting with src-family tyrosine kinases. MIRRs can include, but are not limited to, B cell antigen receptors (BCR), T cell antigen receptors (TCR), and Fc receptors. Examples of Fc receptor MIRR include FcεR and FcRγ. Other examples of receptor types include members of the Ig superfamily and lectin receptors. A cell surface receptor may be functionally linked to an amplifier molecule by an adapter molecule. Eventually activation of the amplifier molecule results in the modulation of downstream effector molecules. Signal transduction often results in changes in gene transcription and leads to a change in the activation state of the cell.

For any receptor, there are mechanisms that regulate the activation of signal transduction pathways, as well as mechanisms that extinguish those signaling pathways. Responding appropriately to environmental signals requires that both positive and negative regulation proceed in an ordered manner (Thomas (1995) J. Exp. Med. 181:1953). In the case of lymphocyte antigen receptors and mast cell and basophil IgE receptors, antigen recognition results in the activation of intracellular protein tyrosine kinases, particularly members of the Src family kinases, as well as the ZAP-70/syk kinases. Signal transduction via these receptors results in the phosphorylation of critical tyrosines within the cytoplasmic

domains of the TCR CD3/ζ chain complex, the α and β chains of the BCR, and the β and γ chains of FcεRI.. The critical target tyrosines are located within a sequence motif termed the immunoreceptor tyrosine-based activation motif (ITAM). ITAM sequences are critical for the activation of immune cells.

Negative regulation of immune cells also plays a key role in immunity, resulting in normal resolution of responses. However, the mechanisms which terminate signaling are not well understood. Immune complexes consisting of antigen and IgG antibodies are known to be potent inhibitors of humoral immune responses that can lead to allergic responses (Chan et al., *Immunology* 21:967-981, 1971 and Kohler et al., *J. Immunol.* 119:1979-1986, 1977). Immune complex mediated inhibition of antibody production has been shown to depend on the interaction between an IgG Fc receptor (FcγRIIB1) and a B lymphocyte antigen receptor (BCR) on a B cell (Phillips et al., *J. Immunol.* 130:602-606, 1983). Mutational analysis of a gene encoding an FcγRIIB1 has revealed that a 13 amino acid motif in the cytoplasmic domain, is required for negative signaling (Muta et al (1994) Nature 368:70; Amigorena et al (1992), *Science* 256:1808-1812; and Fridman et al., *Immunological Reviews* 125:49-76, 1992).

Hematopoietic cells have a variety of functions including inflammatory and immune response functions. Many diseases involve cells derived from hematopoietic cell lineage including hyperimmune diseases, immune deficiency diseases, autoimmune diseases, cancer and allergic responses. Currently, therapies for treatment of abnormal inflammation or immune response can cause unwanted side effects.

Thus, there remains a continuing need for the identification and development of reagents capable of regulating hematopoietic cell function in an animal by treating the cause rather than symptoms of such function. In particular, there is a need for products and processes that permit the effective regulation of specific cellular molecules that will allow the implementation of predictable controls of hematopoietic cell function, thus enabling the treatment of various diseases that are caused by hematopoietic cell dysfunction.

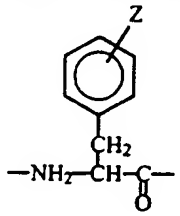
Summary

The present invention relates to compositions and processes for regulating various hematopoietic cell functions. The heretofore unappreciated and unrecognized interactions between particular molecules in certain signal transduction pathway of different hematopoetic cells has resulted in the elucidation of products and processes that permit regulation of cells, exemplary of which are the B cells, T cells, NK cells, basophils and mast cells. Moreover, the present invention permits regulation and treatment of various medical disorders including

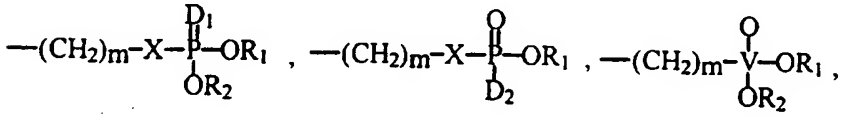
allergic responses, autoimmune diseases, inflammatory responses, immunodeficiency diseases, immunoproliferative diseases.

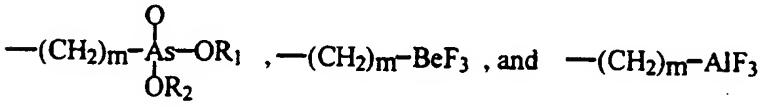
One aspect of the invention relates to a compound comprising an ITIM motif represented in the general formula: (I/V)X_1YX_2X_3(L/V) wherein, I represents isoleucine or a mimetope thereof, V represents valine or a mimetope thereof, each of X_1, X_2 and X_3 independently represents any amino acid or mimetope thereof, Y represents a tyrosine, phosphotyrosine or a mimetope thereof, and L represents leucine or mimetope thereof. In preferred embodiments, X_1 represents a Gly, Ala, Val, Ile, Leu, Ser, Thr, Cys, Glu, Asp, Lys, Arg or His; X_2 represents a Gly, Ala, Val, Ile, Leu, Ser, Cys or Thr; and X_3 represents a Gly, Ala, Val, Ile, Leu, Ser, Cys, Thr, Met, Asn, Gln, Glu or Asp. In more preferred embodiments, X_1 represents a Val, Ile, Leu, Ser, Thr, Asp or His; X_2 represents an Ala, Val, Ser or Thr; and X_3 represents a Leu, Ile, Thr, Met, Gln, Glu or Asp. Exemplary ITIM motifs making up compounds of the present invention include VTYAQL, IVYTEL, VTYTQL, IVYTEL, VTYAQL, ILYTEL, VTYSMV, VTYTTL, VTYSTV, VIYSDL, and ITYAEL, and such motifs can be found in the preferred peptides GAENTITYSLLMH, TAENTITYSLLKH, EQDPQEVTYAQLN, TPPTDIIVYTELP, EQDPQEVTYTQLN, TPPTDIIVYTELP, EQDPEEVTYAQLD, TPPTDTILYTELP, MSEQEVTYSMVRF, MSEQEVTYTTLRF, MSEQEVTYSTVRF, and MDNQGVIYSDLNL.

In many embodiments, it will be desirable to provide the active site tyrosine (Y) as a non-hydrolyzable phosphotyrosine analog. For example, in preferred embodiments Y is represented by the general formula:



,where Z is selected from a group consisting of





where m is zero or an integer in the range of 1 to 6; X is absent or represents O, S, or N; D_1 represents O or S; D_2 represents N_3, SH_2, NH_2, or NO_2; and R_1 and R_2 each independently

represent hydrogen, a lower alkyl, or a pharmaceutically acceptable salt, or R_1 and R_2 taken together with the O-P-O, O-V-O or O-As-O atoms to which they are attached complete a heterocyclic ring having from 5 to 8 atoms in the ring structure.

In certain embodiments at least a portion of the ITIM motif is derived from naturally occurring amino acid residues. However, the present invention also contemplates that all or only a portion of the ITIM motif can be a peptidomimetic. For instance, all or a portion of peptide backbone of the ITIM motif can be derived as a peptidomimetic portion is selected from the group consisting of a retro-inverso peptidomimetic, a retro-enatio peptidomimetic, a trans-olefin peptidomimetic and a phosphonate peptidomimetic.

In preferred embodiments, the ITIM motif is present in a peptidyl portion of the subject compound, which peptidyl portion has a molecular weight in the range of 750 daltons to 7500 daltons, though more preferably in the range of about 750 to 5000 daltons, and even more preferably in the range of about 750 to 2500 daltons. Such molecular weights for any peptide will generally correspond to lengths of 6 to 60 amino acids, though peptides of 6, 7, 8, 9, 10, 11, 12, 13, 15, and 20 amino acid residues are most preferred.

In yet other embodiments, the ITIM motif is covalently linked by one or more peptide bonds (e.g., is part of a single polypeptide chain) to no more than 50 amino acid residues, in addition to the ITIM motif, which are identical to a polypeptide in which the ITIM motif naturally occurs, and more preferably no more than 40, 30 or 25 amino acid residues.

In still other embodiments, the ITIM motif is part of a fusion protein including a second amino acid sequence unrelated to a protein in which the ITIM motif naturally occurs.

In preferred embodiments, compounds including an ITIM motif binds to SH2 domains of a signal transduction protein, e.g., such as a protein tyrosine phosphatase, ITIM-p160 or ITIM-p70. For example, the ITIM motif can bind to PTP1C and/or PTP1D. In such fashion, the compound can modulate, e.g., inhibit or potentiate/activate, a signal transduction acitivity of the cellular component. In preferred embodiments, the ITIM compound regulates hematopoietic cell function by altering the enzymatic activity of an ITIM-binding protein, such as by altering the signal transduction pathway of an MIRR. Preferred ITIM compounds of the present invention bind to PTP1C and activate its phosphatase activity. ITIM-binding proteins are proteins which bind the core ITIM peptide in a phosphotyrosine-dependent manner, such as by recognition of the phosphotyrosine by an SH2 domain.

In other preferred embodiments, the ITIM compound competitively inhibits binding of a PTPase, e.g., PTP1C or PTP1D, with a phosphorylated ITIM sequence the cytoplasmic domain of CD22, NKIRp58, Ly49 or Fcγ receptors.

In preferred embodiments, the ITIM compounds of the present invention are able to modulate second messenger generation in a hematopoetic cell, e.g., alter the phosphorylation of, for example, such signal transduction proteins as Zap70, p72syk, lyn, Vav, TCRz, FceR, Iga or Igb subunit of BCR; and/or increase (mobilize) intracellular free calcium from internal stores or the extracellular mileau. For example, an effective amount of an ITIM compound can reduce the extent of intracellular calcium mobilization resulting from B cell receptor cross-linking.

Another aspect of the present invention provides a pharmaceutical preparation comprising the ITIM compounds above in a physiologically acceptable carrier, and in a therapeutically-effective amount useful for modulating an intracellular signaling pathway in cells in of an animal being treating.

Still another aspect of the invention provides an ITIM mimetic which binds to an SH2 domain of PTP1C, activates the PTP1C phosphatase activity, and competitively inhibits binding of with an ITIM motif of any one or more of CD22, NKIRp58, Ly49 and Fcγ receptors.

Other embodiments of the present invention provide a soluble peptide fragment of a negative regulatory receptor selected from the group consisting of CD22, NKIRp58, Ly49 and Fcγ receptors (e.g., FcγRI, FcγRII, FcγRIII), which peptide includes an ITIM motif. In preferred embodiments: the peptide has a molecular weight in the range of 750 to 7500 daltons; the peptide binds to an SH2 domain of PTP1C and allosterically regulates the tyrosine phosphatase activity of the PTP1C.

Yet another aspect of the present invention provides a method for modulating an an intracellular signaling pathway in a cell comprising altering an activity of an ITIM-binding protein involved in the intracellular signaling pathway by contacting the cell with a compound comprising an ITIM motif represented by the general formula:

$$(I/V)X_1YX_2X_3(L/V)$$

wherein, I represents isoleucine or a mimetope thereof, V represents valine or a mimetope thereof, each of X_1, X_2 and X_3 independently represents any amino acid or mimetope thereof, Y represents a tyrosine, phosphotyrosine or a mimetope thereof, and L represents leucine or mimetope thereof. The cell can be, for example, a B cell, a mast cell, a basophil, a T cell, and/ or a natural killer (NK) cell. The cell can be treated *ex vivo* or *in vivo*.

In a preferred embodiment, the method is used to modulate immune responses, inflammatory responses, blood clotting initiation, release of histamine, destruction of virally infected cells, and/or destruction of infectious agents. For example, the subject method can

be used to regulate the production and/or release of molecules such antibodies, cytokines, chemokines, complement, prostglandins, leukotrienes and inflammatory mediators.

The subject method can used to treat or prevent such disease states as immunoproliferative diseases, immunodeficiency diseases, cancers, autoimmune diseases, infectious diseases and allergic responses. The method protects an animal from a disease involving autoantibody production or involving IgE-induced basophil degranulation.

In preferred embodiments, the method regulates hematopoietic cell function by inhibiting the activity of an MIRR, as for example by causing stimulation, e.g., ITIM-dependent acitivation, of the specific phosphatase activity of PTP1C.

In yet another embodiment, the present invention provides a method for regulating hematopoietic cell function by contacting a hematopoietic cell with an effective amount of a regulatory reagent capable of altering the activity of a regulatory molecule selected from the group consisting of PTP1C, PTP1D, ITIM-p160 and ITIM-p70. Such regulatory molecules include ITIM mimetics as described herein, as well as general phosphotyrosine peptides such as pYXXL and pYXXV, and bispecific antibodies capable of crosslinking a stimulatory receptor protein with an inhibitory ITIM-containing receptor protein. In preferred embodiments, the method alters such hematopoietic cell function as selected from the group consisting of immunoproliferative diseases, immunodeficiency diseases, cancers, autoimmune diseases, infectious diseases and allergic responses. For example, the method can be used to regulate the production and/or release of molecules such as antibodies, cytokines, chemokines, complement, prostglandins, leukotrienes and inflammatory mediators. The cell can be, for example, a B cell, a mast cell, a basophil, a T cell, and/ or a natural killer (NK) cell. The cell can be treated *ex vivo* or *in vivo*. The method can be used to protect an animal from a disease involving autoantibody production or involving IgE-induced basophil degranulation.

In a preferred, the subject method regulates hematopoietic cell function by inhibiting the activity of a MIRR, e.g., such as by stimulating the specific phosphatase activity of PTP1C or PTP1D.

Yet another aspect of the present invention relates to therapeutic compositions that, when administered to an animal in an effective amount, are capable of regulating signal transduction in the cells of that animal. A therapeutic composition of the present invention is useful for the treatment of any disease caused in part by abnormal signal transduction in a cell. Such diseases include cancer, autoimmune disease, immunodeficiency diseases, immunoproliferative diseases, allergic responses, and inflammatory responses. A therapeutic composition of the present invention is also useful in the regulation of an immune response

during medical treatments, such as transplantation of organs or skin. Autoimmune diseases can include, for example, systemic lupus, myasthenia gravis, rheumatoid arthritis, insulin dependent diabetes mellitus and experimental allergic encephalomyelitis. Immunodeficiency diseases can include, for example, human AIDS, severe combined immunodeficiencies and hypogammaglobulinemia. Immunoproliferative diseases can include, for example, lymphomas and leukemias. In addition, a therapeutic composition of the present invention is useful for the treatment of all forms of cancer, including tumor formation.

The present invention also makes available a method to identify agents, such as other ITIM mimetics, capable of altering a signal transduction activity of an SH2-containing protein, the method comprising: (a) forming a reaction mixture including (i) a compound, such as a peptide, comprising an ITIM motif, (ii) a target protein including at least one SH2 domain which specifically binds the ITIM motif by the SH2 domain, and (iii) a test agent; and (b) detecting interaction of the target protein with the ITIM motif, wherein a statistically significant change in the interaction of the target protein in the presence of the test agent, relative to the level of interaction in the absence of the test agent, indicates an activity of the test agent for altering an ITIM-dependent signal transduction activity of the SH2-containing target protein. In preferred embodiments, the target protein is a PTPase, such as PTP1C or PTP1D, or other ITIM-binding protein.

Detecting interaction of the target protein with the compound can be by a variety of formats, including measuring the level of enzymatic activity of the target protein, or detecting the direct interaction of the target protein with the compound. In certain embodiments, the the reaction mixture is a whole cell, and detecting interaction of the target protein with the compound comprises measuring the level of phosphorylation of one or cellular proteins. In other embodiments, the reaction mixture is a whole cell or cell lysate and detecting interaction of the target protein with the compound comprises measuring the level of intracellular calcium. In preferred embodiments, the reaction mixture is derived by recombinant expression of the compound in a cell.

Detecting interaction of the target protein with the ITIM compound can be carried out by any of a number of techniques that will be appreciated in the art. For example, in preferred embodiments, the detection can comprise measuring one or more of (i) the level of phosphorylation of a cellular protein, (ii) the level of intracellular Ca^{2+}, (iii) the level of a diacylglyceride, (iv) the level of an inositol phosphate, (v) the level of expression of an immediate early (cIE) gene product, such as myc, fos or jun.

Similar methods can be used to detect agents which alter other novel therapeutic targets identified herein. For instance, the present invention also provides a method for identifying agents capable of altering the CD19-mediated signal transduction comprising:(a)

forming a reaction mixture including (i) a PTP1C phosphatase, (ii) a phosphorylated CD19 protein or PTP1C substrate derived therefrom, and (iii) a test agent; and (b) detecting interaction of PTP1C amd CD19. A statistically significant change in the interaction of PTP1C and CD19 in the presence of the test agent, relative to the level of interaction in the absence of the test agent, indicates an activity of the test agent for altering a signal transduction activity of the CD19 protein.

The present invention also provides kits for performing such drug screening assays. An exemplary kit for identifying compounds capable of regulating hematopoietic cell function comprises a PTP1C or PTP1D phosphatase, an ITIM mimetic capable of binding and activating the PTP, a PTP substrate, and a putative regulatory compound.

Still another aspect of the present invention provides a method to identify a treatment for abnormal hematopoietic cell function, comprising: (a) isolating basophils from a patient; (b) contacting the basophils with a ligand selected from the group consisting of Fab' IgG antibody and whole IgG antibody; and (c) diagnosing abnormal hematopoietic cell function by determining PTP1C activity in the basophils after the step of contacting with the ligand.

Other aspects and embodiments of the present invention will become obvious to one of ordinary skill in the art after consideration of the drawings and detailed description provided below.

Brief Description of the Figures

Figure 1 shows the sequence alignment between inhibitory receptors. The GenBank accession numbers for the sequences used in this paper are: human p58.183: U24074, human p58.EB6: U24075, human p70: L41269, mouse Ly49A: M25812, mouse Ly49C: U10304, mouse Ly49G4: U10093, human CD22b: X59350, mouse CD22b: L16928, human FcgRIIB1: M31935, mouse FcgRIIB1: M17515, NKG2A: X54867. Amino-acid numbers corresponding to the stretches are indicated.

Figure 2 shows inhibition of BCR mediated Ca2+ mobilization by the PI 3-kinase inhibitor Wortmannin. Ca^{2+} mobilization in A20 cells stimulated with intact (- - -) rabbit anti-mouse Ig antibody, or $F(ab')_2$ fragments in the presence (——) or absence (—) of 10nM Wortmannin. A20 cells were loaded with Indo-1 AM (Molecular Probes) and subsequently stimulated with either $F(ab')_2$ (12 μg/ml) or intact (20 μg/ml) rabbit anti-mIg, and $[Ca^{2+}]_i$ was monitored with a flow cytometer for 15 min (model 50H, Ortho Diagnostic Systems). The mean $[Ca^{2+}]_i$ (panel A) and percent of cells that responded (panel B) was evaluated with an appended data acquisition system and the MultiTIME software (Phoenix Flow Systems).

Figure 3 shows that CD19 is required for prolonged BCR mediated Ca2+ mobilization. Cell surface expression of CD19 on untransfected J558Lμm3CD45+ (panel A), and J558Lμm3CD45+ transfected with human CD19 (panel B). Ca^{2+} mobilization in response to antigen (NP9-BSA, 3μg/ml) in untransfected (panel C) or hCD19 transfected cell lines (panel D). Cell surface expression of hCD19 was detected by staining cells with analysis by flow cytometry (FACScan, Becton Dickinson). Ca^{2+} mobilization was analyzed as in Figure 2.

Detailed Description Of The Invention

The signal transduction pathways engaged upon receptor-ligand interaction represent the key mechanism by which cellular responses are controlled. The signals transduced by cell surface receptors may be either stimulatory or inhibitory depending upon the intracellular cascade of events which is initiated. There are numerous points at which a signal transduction pathway can be regulated. Examples include regulation of the interactions of ligands with receptors, interaction of receptor/transducer molecules with adapter molecules, interactions of adapter molecules with amplifier molecules, interactions of amplifiers with other potential targets, or regulation of the generation of second messengers.

For any given receptor, there are mechanisms that regulate the activation of signal transduction pathways, as well as mechanisms that extinguish those signaling pathways. Responding to environmental signals requires that both positive and negative regulation proceed in an ordered manner. Accordingly, the mechanisms that terminate signaling are equally important as the stimulatory signals. The inability to properly extinguish activation cascades may result in inappropriate responses giving rise to various disease states. The present invention provides for agents which are useful in the modulation, e.g. either to potentiate or inhibit the activation of hematopoetic cells by altering the balance of stimulatory and inhibitory signal transduction. In addition to therapeutic applications, the present invention also relates to the development of drug screening assays useful in the identification of other cell regulatory compounds.

In particular, various aspects of the present invention derive from the elucidation of certain mechanisms by which signal transduction from MIRRs and the like are down regulated. For example, as disclosed herein, coligation of the Fc receptor on B cells, e.g., Fcγ RIIB1, with the B cell antigen receptor (BCR) leads to abortive BCR signaling through a mechanism which includes recruitment of the protein tyrosine phosphatase PTP1C by the Fcγ R. The recruitment and activation of PTP1C to the receptor oligomer is mediated, as demonstrated below, by binding between the carboxyl-terminal Src homology 2 (SH2) domain of PTP1C and a phosphotyrosine motif, termed an immunoreceptor tyrosine-based

inhibitory motif (ITIM), present in the cytoplasmic tail of the FcγR. Thus, PTP1C is an effector of BCR-FcγRIIB1 negative signal cooperation and represents a potential therapeutic target for up- or down-regulation of humoral immune responses both *in vitro* and *in vivo*.

Moreover, as is disclosed herein, inhibition of natural killer (NK) cell cytotoxic programs results from the recruitment of PTP1C and/or PTP1D by ITIM sequence(s) present in the NK MHC class I protein receptors (NKIR). The effector functions initiated by stimulation of the CD3/TCR complex are also demonstrated to be inhibited by a PTP1C/1D-dependent mechanism. Accordingly, recruitment of and/or activation of specific phosphatases by ITIM sequences is apparently a mechanism shared by several different pathways as a means for downregulating receptor function with.

The core peptide sequence of the ITIM which is critical to activation of PTP1C and PTP1D is also disclosed. The optimal sequence for PTP1C activation, contrary to the general teachings in the art concerning SH2 domain specificity, requires amino acid residues both C-terminal and N-terminal to the phosphotyrosine.

Still other aspects of the present invention derive from the discovery of novel interactions beyond the ITIM/PTP interactions. For instance, the appended examples describe that the BCR co-receptor CD19 is an apparent substrate for PTP1C. Dephosphorylation of CD19 by FcγR-activated PTP1C leads to diminished association of CD19 with PI-3K and subsequent failure of BCR-mediated Ca^{2+} mobilization. Accordingly, the dephosphorylation reaction of CD19 by PTP1C is another potential therapeutic target for developing agents which modulate B cell activation and/or inhibition.

Other potential therapeutic targets are the ITIM p160 and ITIM p70 interactions described below. Like PTP1C, each of these proteins demonstrate binding to phosphorylated ITIM peptides indicating the presence of an ITIM-binding motif.

It will be evident that the present invention contemplates a number of dfferent regulatory reagents, ranging from ITIM mimetics to agents which modulate dephosphorylation of CD19 or other signal transduction protein by inhibiting an ITIM activated phosphatase. According to the present invention, a regulatory reagent can regulate cell function by, for example: altering the interaction between a signal transduction molecule and an amplifier, or target molecule; altering the binding between the signal transduction molecule and its target molecule; altering the enzymatic activity of a signal transduction molecule, or by modulating the phosphorylation of the signal transduction or other target molecule. As used herein, "altering the binding" can refer to altering the affinity of one molecule for another, blocking the sites of binding between two molecules, or interfering with the delivery of a molecule to the area of another molecule or allosterically altering a

molecule so that it has either enhanced or diminished binding abilities. The term "modulation" of signal transduction pathways is meant to include either agonistic or antagonistic effects on cellular activation. As would be understood by one of skill in the art, any alteration in a signal transduction pathway would be indicated by a statistically significant change in an assay result in the presence of a putative regulatory compound relative to a result obtained in the absence of the compound.

In one aspect the compounds of the present invention and compositions in which they are employed, are particularly advantageous in that they are useful for regulating a wide variety of cellular functions by regulating different stages of signal transduction in a cell. As described in further detail below, the subject compounds can be employed to effect such cellular events as proliferation, differentiation and cell death, as well as secretion and metabolic (both anabolic and catabolic) activities, in both cell culture and in vivo therapies. Moreover, another aspect of the invention provides for methods to identify additional compounds capable of regulating signal transduction in a cell.

The compounds of the present invention are capable or regulating the activation of hematopoetic cells. According to the present invention, hematopoietic cells include erythrocytes (red blood cell) and leukocytes (white blood cell). Leukocytes include neutrophils (i.e., polymorphonuclear leukocytes), basophils, eosinophils, mast cells, megakaryocytes, monocytes, lymphocytes, natural killer (NK) cells and macrophages. Particularly preferred hematopoietic cells of the present invention include, NK cells, basophils, B lymphocytes (B cells) and T lymphocytes (T cells). In addition, the use of the subject compounds is capable of regulating red blood cell growth and or differentiation.

One aspect of the invention concerns a peptide or peptide analog capable of regulating the activity of a hematopoetic cell. In particular, the peptides of the present invention include the general or core amino acid motif (hereinafter "ITIM" motif) represented by the general formula (I/V)XYXX(L/V), in which each of I represents isoleucine or a mimetope thereof, V represents a valine or a mimetope thereof, each X independently represents any amino acid or mimetope thereof, Y represents a tyrosine, a phosphotyrosine or a mimetope thereof, and L represents a leucine or mimetope thereof. In preferred embodiments the tyrosine residue is phosphorylated. As demonstrated in the appended examples, an isolated ITIM motif is sufficient, e.g., contains the necessary structural information, for modulating a variety of signal transduction processes involving hematopoetic receptors.

In one embodiment, the subject ITIM peptide comprises an ITIM sequence represented by (V/I)XYXX(V/L) such as VTYAQL, IVYTEL, VTYTQL, IVYTEL, VTYAQL, ILYTEL, VTYSMV, VTYTTL, VTYSTV, VIYSDL, or ITYAEL, wherein each V represents a valine or mimetope thereof, T represents a threonine or mimetope thereof, Y

represents a phosphotyrosine or mimetope thereof, A represents an alanine or mimetope thereof, Q represents a glutamine or mimetope thereof, L represents a leucine or mimetope thereof, I represents an isoleucine or mimetope thereof, E represents a glutamic acid or mimetope thereof, S represents a serine or mimetope thereof, M represents a methionine or mimetope thereof, and D represents an aspartic acid or mimetope thereof. In preferred embodiments the tyrosine residue is phosphorylated. In other embodiments ITIM peptides comprise an ITIM sequence represented by one of EQDPQEVTYAQLN, TPPTDIIVYTELP, EQDPQEVTYTQLN, TPPTDIIVYTELP, EQDPEEVTYAQLD, TPPTDTILYTELP, MSEQEVTYSMVRF, MSEQEVTYTTLRF, MSEQEVTYSTVRF, or MDNQGVIYSDLNL. Preferred ITIM peptides are derived from NKIRp58 or Ly49. In preferred embodiments the tyrosine residue is phosphorylated.

In another embodiment, the subject ITIM peptide includes a core ITIM sequence represented in the general formula IXYXXL, as for example ITYSLL. Preferred ITIM peptides are represented by either of TAENTITYSLLKH or GAENTITYSLLMH, in which each E represents a glutamic acid or mimetope thereof, G represents glycine or a mimetope thereof, A represents alanine or a mimitope thereof, N represents asparagine or a mimetope thereof, each T represents threonine or a mimetope thereof, I represents isoleucine or a mimetope thereof, Y represents tyrosine or phosphotyrosine or a mimetope thereof, S represents serine or a mimetope thereof, each L represents leucine or a mimetope thereof, K represents lysine or a mimetope thereof, M represents methionine or a mimetope thereof, and H represents histidine or a mimetope thereof. In preferred embodiments the tyrosine residue is phosphorylated. Preferably, ITIM peptides are derived from Fc receptor sequences, and more preferably B cell Fc receptors. Preferred ITIM peptides are derived from an FcγRII and more preferably from an FcγRIIB1.

In preferred embodiments, the tyrosine residue of the ITIM motif is phosphorylated, or is a phosphotyrosine analog, e.g. as described below. Although forms in which the tyrosine residue is not phosphorylated are also contemplated.

ITIM agonists and ITIM antagonists (e.g., "ITIM mimetics" or "mimetopes") are molecules which are able to mimic or inhibit, respectively, the transduction of a signal by an ITIM sequence occurring in a cellular protein. Particularly as competitive inhibitors of ITIM mediated activation of PTP1C or PTP1D. The agonism or antagonism of an ITIM-dependent signal can arise from, for example, the ITIM mimetic allosterically activating an ITIM-binding protein, e.g., altering its enzymatic and/or binding specificity profile, or by competively inhibiting the binding of an ITIM-binding protein with a naturally occurring ITIM sequence. An ITIM mimetic is bioactive if it is capable of regulating the production of

antibodies, cytokines, chemokines, complement, prostglandins, leukotrienes and other inflammatory mediators by a cell involved in inflammatory and immune responses.

For instance, ITIM mimetics of the present invention are capable, through modulating signal transduction, of ultimately regulating the phosphorylation of intracellular substrates, the production of second messenger molecules, and the activation of gene transcription. Target molecules can include, but are not limited to, phosphatidylinositol 3-kinase (PI-3K), Grb 2, sos, P21rasGAPase-activating protein and associated P190 and P62 protein, phospholipases such as PLCγ1 and PLCγ2, Shc, and the MAP kinases, including the Janus (JAK) family of kinases. In one embodiment the compound of the present invention is capable of modulating the phosphorylation of the z chain of the TCR, Iga or Igb subunits of the BCR, Zap 70, p72syk, Lyn, or Vav. Accordingly, ITIM mimetics of the present invention can be used to modulate the production of such natural products as C5a, interleukin-8 (IL-8), monocyte chemotactic protein 1α (MIP1α), monocyte chemotactic protein 1β (MIP1β), monocyte chemoattractant protein 1 (MCP-1), monocyte chemoattractant protein 3 (MCP-3), platelet activating factor (PAF), N-Formyl-methionyl-leucyl-[3H]phenylalanine (FMLP), leukotriene B4 (LTB4), LTC4, gastrin releasing peptide (GRP), RANTES, eotaxin, lymphotactin, IP10, I-309, ENA78, GCP-2, NAP-2, MGSA/gro, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1), insulin, IgE/antigen, IgG/antigen, IgA/antigen, major histocompatibility (MHC) protein, peptide, superantigen, antigen, interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-7 (IL-7), interleukin-9 (IL-9), interleukin-10 (IL-10), interleukin-11 (IL-11), interleukin-13 (IL-13), interleukin-14 (IL-14), interleukin-15 (IL-15), interleukin-16 (IL-16), granulocyte colony stimulating factor (G-CSF), granulocyte-megakaryocyte colony stimulating factor (GM-CSF), colony stimulating factor-1 (CSF-1), erythropoietin (EPO), tumor necrosis factor (TNF), Fas, interferon-γ (IFN-γ), interferon-α/β (IFN-α/β), OSM, Lif, CNTF, prolactin, neuropeptides, catecholamine, Ach, histamine, peptide eosinophil chemotactic factor, TXA2, or PGD2.

In one embodiment of the invention, the subject ITIM mimetics are capable of competing with the cytoplasmic domains of a cell surface receptor for the binding of protein tyrosine phosphatases. In a preferred embodiment, mimetics are capable of competing for the binding of an SH2 containing protein tyrosine phosphatase (PTP) with a portion of the cytoplasmic domain of a receptor, such as one of the group of FcγR, natural killer cell receptor for MHC class I (for example, NKIRp58 or Ly 49), CD22, IL2βR, IL-3βR, EPOR, and c-kit. In a preferred embodiment the peptides of the present invention are capable of competing for the binding of an SH2 containing PTP with a portion of the cytoplasmic domain of an FcγR or Ly 49 or NKIRp58R. In particularly preferred embodiments the

subject peptides are capable of competing for the binding of an SH2 containing PTP with a portion of the cytoplasmic domain of an FcγRI, FcγRII, FcγRIII, Ly49, or NKIRp58R, more preferably with FcγRIIB1 In preferred embodiments the PTP is one of PTP1C or PTP1D.

In another embodiment, the subject ITIM peptides are encoded by a nucleic acid which hybridizes under stringent conditions with a nucleic acid encoding an ITIM portion of the cytoplasmic domain of an FcγR, MHC class I R, CD22, IL2βR, IL3βR, or EPOR. In particularly preferred embodiments nucleic acids encoding the subject peptides are capable of specifically hybridizing to a nucleic acid comprising the ITIM portion of the cytoplasmic domain of an FcγRI, FcγRII, FcγRIII, Ly49, or NKIRp58R, more preferably with FcγRIIB1.

In a preferred embodiment, ITIM mimetics of the present invention are capable of modulating the interaction of a PTP with CD19, resulting in changes in the phosphorylation pattern of CD19. In a particularly preferred embodiment, through activation of a phosphatase, e.g. PTP1C or PTP1D, the subject compounds lead to the dephosphorylation of tyr 484 and/or 515 on CD19. In one embodiment of the present invention an ITIM mimetic leads to a modulation in the activation of PI3 kinase. In another embodiment, an ITIM mimetic is capable of modulating the activation of PLCγ. In still another embodiment the subject mimetics lead to a modulation in intracellular second messenger generation, for example Ca^{2+} influx from outside the cell, by causing a inhibiting the interaction of CD19 with PI3kinase activity. In another embodiment a subject ITIM peptide is capable of modulating second messenger generation by modulating the production of IP3 and thus the release of Ca^{2+} from intracellular stores. Methods of detecting second messenger generation, and thus effects of the subject ITIM peptides, are well known in the art. Examples of possible detection methods are described in the instant specification and in the appended Examples.

In another embodiment an ITIM mimetics of the present invention is capable of modulating the activity of Grb2 or sos, preferably by binding to an SH2 containing PTP.

In yet another embodiment, the present invention relates to ITIM binding molecules. As used herein an ITIM binding molecule is capable of binding, via a phosphotyrosine residue to an ITIM peptide or mimetic. In preferred embodiments an ITIM peptide or mimetic binds to SH2 domains. In a more preferred embodiment an ITIM mimetic binds an SH2 containing tyrosine phosphatase in a manner such that the peptide is capable of regulating the activity of the phosphatase. In a particularly preferred embodiment an ITIM peptide activates a PTP. In still another embodiment, an ITIM mimetic is of a size and nature that enables the compound to be bound by the carboxy terminal SH2 domain of a PTP. In other embodiments PTP1D, p160, or p70 are ITIM binding molecules.

In a preferred embodiment an ITIM mimetic of the present invention binds to and regulates the activity of a PTP present in hematopoetic cells, for example PTP1C or PTP1D. In a more preferred embodiment the mimetics of the present invention bind to PTP1C or PTP1D SH2 domain resulting in its regulation of phosphatase activity by an allosteric mechanism. In a particularly preferred embodiment an ITIM of the present invention binds to the carboxy terminal SH2 domains resulting in an increase in the phosphatase activity of the enzyme. Such a modulation in phosphatase activity may be measured using any number of techniques, including measuring changes in the level of phosphorylation of a known natural or synthetic substrate, such as phosphorylated lyn peptide as described in the appended Examples.

In another embodiment, an ITIM peptide of the present invention is capable of stimulating the specific activity of PTP1C. As used herein, the term "specific activity" refers to the rate at which a PTP1C molecule can dephosphorylate a phosphorylated substrate molecule, such as phosphorylated CD19, Syk, Lyn, Fyn or PI-3 kinase. The rate of specific activity can be measured by, for example, the rate at which PTP1C dephosphorylates a phosphorylated substrate molecule. Preferably, an ITIM peptide of the present invention is capable of stimulating the specific activity of PTP1C between about 2-fold and about 10-fold, and more preferably about 5-fold. Stimulation of specific activity is seen when the peptide is incubated with PTP1C in the presence of phosphorylated Fyn peptide as described in detail in Example 5.

A regulatory reagent of the present invention regulates hematopoietic cell function by altering the enzymatic activity of a signal transduction molecule, preferably by stimulating the enzymatic activity of a signal transduction molecule. In addition, a regulatory reagent of the present invention regulates hematopoietic cell function by modulating the activity of an activating receptor including an MIRR, a lectin receptor, or an Ig superfamily receptor. In preferred embodiments the subject ITIMs regulate signaling via the BCR, TCR,FcεR, a cytokine receptor, or CD16. More preferably a peptide of the present invention modulates the signals transduced by one of these receptors by modulating a signal transduced via an inhibitory receptor, such as, CD22, FcεRI, FcγRIII, NK1Rp58, Ly49, IL2Rb, IL3Rb, EPOR, or c-kit. In particularly preferred embodiments a peptide of the present invention inhibits via a CD22, FcγRIII, NK1Rp58, Ly49, IL2Rb, IL3Rb, EPOR, or c-kit, molecule.

One embodiment of the present invention includes a regulatory reagent that is capable of regulating the activity of one or more of the signal transduction molecules phosphotyrosine phosphatase 1C (PTP1C), PTP1D, ITIM-p160 and/or ITIM-p70, in a cell. Suitable methods for regulating PTP1C, PTP1D, ITIM-p160 and/or ITIM-p70 activity in a cell include altering the enzymatic activity of the molecule; altering the binding ability of the molecule to its

substrate; altering the concentration of the molecule in a cell; and/or phosphorylating the molecule. In preferred embodiments a subject ITIM is capable of modulating the binding of a PTP to its substrate. In other preferred embodiments an ITIM modulates the binding of ITIM p160 or ITIMp70 to upstream or downstream elements in a signaling cascade. In a particularly preferred embodiment an ITIM peptide competes for the binding of ITIM p160 or p70 with the cytoplasmic domain of a negative regulatory receptor, such as CD22, FcγR, or MHC class I R.

One embodiment of the present invention pertains to a composition that is capable of altering the enzymatic activity of a PTP. Preferably such reagents include compounds that are capable of binding to a src homology region of a PTP, preferably a src homology region 2 (SH2) region of PTP, and more preferably a carboxyl SH2 domain of a PTP. In another embodiment a peptide of the present invention binds to the catalytic region of a PTP. In preferred embodiments the PTP is at least one of PTP1C or PTP1D.

Another embodiment includes a regulatory reagent that is capable of altering the binding ability of a PTP to its substrate. In a preferred embodiment the PTP is at least one of PTP1C or PTP1D. Preferably such reagents include compounds that are capable of binding to an SH2 domain and/or catalytic domain of a PTP, or phosphotyrosine sites of a substrate of PTP, such as the tyrosine autophosphorylation site on an ITAM region of an MIRR. A preferred tyrosine autophosphorylation site is residue 394 of pITAM of the Igα and Igβ subunits of the B cell receptor. In particularly preferred embodiments such reagents are capable of binding to phosphotyrosines 484 and/or 515 of CD19. The ability of a subject peptide to modulate the binding of a PTP to other substrates is also contemplated.

Another embodiment of the present invention includes a regulatory reagent, or mimetope thereof, that is capable of altering the concentration of PTP1C in a cell. Preferably, such reagents include PTP1C protein or a nucleic acid molecule encoding PTP1C (described in detail below).

Another embodiment of the present invention includes a regulatory reagent, or mimetope thereof, that is capable of altering the activity of ITIM-p70 in a cell. Preferably, such reagents include compounds capable of binding to a portion of ITIM-p70 (see appended Examples) such that the enzymatic or substrate binding activity of the protein is altered. Also preferred are compounds capable of increasing the concentration of ITIM-p70 in a cell, such as a protein or a nucleic acid molecule encoding for ITIM-p70 protein.

According to the present invention, an isolated, or biologically pure peptide, is a peptide that has been removed from its natural milieu. As such, "isolated" and "biologically pure" do not necessarily reflect the extent to which the protein has been purified. An isolated

ITIM containing peptide can be obtained from a natural source, e.g., isolated peptide from a full-length protein, produced using recombinant DNA technology, or chemical synthesis as is well known in the art. Moreover, the peptide can be substituted and/or derivatized, e.g., acetylated, glycosylated, carboxymethylated anchored by myristoylated, prenylated or palmitoylated amino acids. In one embodiment, of the present invention includes an isolated ITIM peptide that has an amino acid sequence that enables the peptide to be bound by PTP1C, PTP1D, ITIM-p160 and/or ITIM-p70. An ITIM peptide of the present invention is of a size that enables the peptide to be bound by at least one binding site of PTP1C, PTP1D, ITIM-p160 and/or ITIM-p70, preferably, at least about 4 amino acid residues, more preferably at least about 5 amino acid residues, and even more preferably at least about 6 amino acid residues. In one embodiment, an ITIM peptide of the present invention has a size of about 13 amino acid residues. In particular, an ITIM peptide of the present invention is capable of being bound by an SH2 domain PTP1C. Preferably, an ITIM peptide of the present invention is capable of being bound by a carboxyl SH2 domain of PTP1C. In preferred embodiments, the peptidyl component of the subject compounds includes, in addition to the ITIM, no more than about 25 amino acid residues of a protein in which an ITIM motif exists, more preferably no more than 10-15, and even more preferably 6 or less. With the exception of certain chimeric ITIM compositions described herein, such as fusion proteins, a preferred composition (especially for ectopic application) includes a peptide comprising ITIM- motif and having a molecular weight in the range of about 750 to 7500 daltons, more preferably from about 1000 to 5000 daltons, and even more preferably in the range of about 1000 to 2500 daltons.

In accordance with the present invention, a "mimetope" refers to any compound that is able to mimic the ability of an isolated compound of the present invention. Indeed, as used throughout this application, the term "peptide", with reference to ITIM motifs, will be understood to also include mimetopes. A mimetope can be a peptide that has been modified to decrease its susceptibility to degradation but that still retains regulatory activity. Other examples of mimetopes include, but are not limited to, protein-based compounds, carbohydrate-based compounds, lipid-based compounds, nucleic acid-based compounds, natural organic compounds, synthetically derived organic compounds, anti-idiotypic antibodies and/or catalytic antibodies, or fragments thereof. A mimetope can be obtained by, for example, screening libraries of natural and synthetic compounds for compounds capable of regulating the activity of a tyrosine kinase as disclosed herein. A mimetope can also be obtained, for example, from libraries of natural and synthetic compounds, in particular, chemical or combinatorial libraries (i.e., libraries of compounds that differ in sequence or size but that have the same building blocks). A mimetope can also be obtained by, for example,

rational drug design. In a rational drug design procedure, the three-dimensional structure of a compound of the present invention can be analyzed by, for example, nuclear magnetic resonance (NMR) or x-ray crystallography. The three-dimensional structure can then be used to predict structures of potential mimetopes by, for example, computer modelling. the predicted mimetope structures can then be produced by, for example, chemical synthesis, recombinant DNA technology, or by isolating a mimetope from a natural source (e.g., plants, animals, bacteria and fungi).

By the terms "amino acid residue" and "peptide residue" it is meant an amino acid or peptide molecule without the -OH of its carboxyl group (C-terminally linked) or the proton of its amino group (N-terminally linked). In general the abbreviations used herein for designating the amino acids and the protective groups are based on recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (see *Biochemistry* (1972) 11:1726-1732). For instance Met, Ile, Leu, Ala and Gly represent "residues" of methionine, isoleucine, leucine, alanine and glycine, respectively. By the residue is meant a radical derived from the corresponding a-amino acid by eliminating the OH portion of the carboxyl group and the H portion of the a-amino group. The term "amino acid side chain" is that part of an amino acid exclusive of the $-CH(NH_2)COOH$ portion, as defined by K. D. Kopple, "Peptides and Amino Acids", W. A. Benjamin Inc., New York and Amsterdam, 1966, pages 2 and 33; examples of such side chains of the common amino acids are $-CH_2CH_2SCH_3$ (the side chain of methionine), $-CH_2(CH_3)-CH_2CH_3$ (the side chain of isoleucine), $-CH_2CH(CH_3)_2$ (the side chain of leucine) or H-(the side chain of glycine).

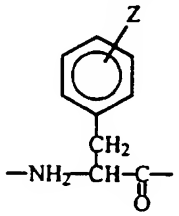
Moreover, for the most part, the amino acids used in the application of this invention are those naturally occurring amino acids found in proteins, or the naturally occurring anabolic or catabolic products of such amino acids which contain amino and carboxyl groups. Particularly suitable amino acid side chains include side chains selected from those of the following amino acids: glycine, alanine, valine, cysteine, leucine, isoleucine, serine, threonine, methionine, glutamic acid, aspartic acid, glutamine, asparagine, lysine, arginine, proline, histidine, phenylalanine, tyrosine, and tryptophan.

However, as is apparent from the contemplation of mimetopes, the term amino acid residue further includes analogs, derivatives and congeners of any specific amino acid referred to herein. For example, the present invention contemplates the use of amino acid analogs wherein a side chain is lengthened or shortened while still providing a carboxyl, amino or other reactive precursor functional group for cyclization, as well as amino acid analogs having variant side chains with appropriate functional groups). For instance, the subject peptidomimetic can include an amino acid analog as for example, b-cyanoalanine, canavanine, djenkolic acid, norleucine, 3-phosphoserine, homoserine, 1-methylhistidine,

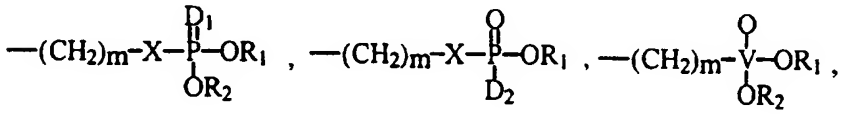
dihydroxyphenylalanine, 5-hydroxy-tryptophan, or 3-methylhistidine. Other naturally occurring amino acid metabolites or precursors having side chains which are suitable herein will be recognized by those skilled in the art and are included in the scope of the present invention.

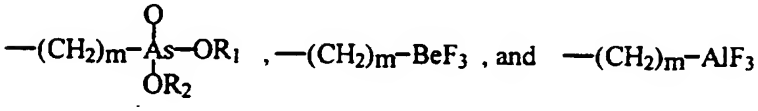
Also included are the D and L stereoisomers of such amino acids when the structure of the amino acid admits of stereoisomeric forms. The configuration of the amino acids and amino acid residues herein are designated by the appropriate symbols D, L or DL, furthermore when the configuration is not designated the amino acid or residue can have the configuration D, L or DL. It will be noted that the structure of some of the compounds of this invention includes asymmetric carbon atoms. It is to be understood accordingly that the isomers arising from such asymmetry are included within the scope of this invention. Such isomers are obtained in substantially pure form by classical separation techniques and by sterically controlled synthesis. For the purposes of this application, unless expressly noted to the contrary, a named amino acid shall be construed to include both the D or L stereoisomers, though it will be understood that the amino acid is preferably the L stereoisomer.

Likewise, such mimetopes can include analogs of the phosphotyrosine. For example, a phosphotyrosine moiety, pTyr, can be represented by the general formula



,where Z is selected from a group consisting of



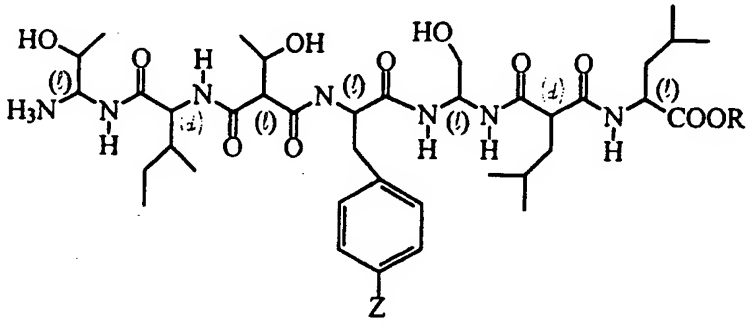


where m is zero or an integer in the range of 1 to 6; X is absent or represents O, S, or N; D_1 represents O or S; D_2 represents N_3, SH_2, NH_2, or NO_2; and R_1 and R_2 each independently represent hydrogen, a lower alkyl, or a pharmaceutically acceptable salt, or R_1 and R_2 taken together with the O-P-O, O-V-O or O-As-O atoms to which they are attached complete a heterocyclic ring having from 5 to 8 atoms in the ring structure. The *para*-substituted phenylalanine is the most preferred. In many embodiments, it will be desirable that the

phosphoTyrosine be a non-hydrolyzable phosphotyrosine analog. In a preferred embodiment, the phosphotyrosine is phosphonodifluoromethyl phenylalanine (F_2Pmp).

Moreover, as is apparent from the present and parent disclosures, mimetopes of the subject ITIM peptides can be provided as non-hydrolyzable peptide analogs. For illustrative purposes, peptide analogs of the present invention can be generated using, for example, benzodiazepines (e.g., see Freidinger et al. in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gama lactam rings (Garvey et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988, p123), C-7 mimics (Huffman et al. in *Peptides: Chemistry and Biologyy*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988, p. 105), keto-methylene pseudopeptides (Ewenson et al. (1986) *J Med Chem* 29:295; and Ewenson et al. in *Peptides: Structure and Function* (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985), b-turn dipeptide cores (Nagai et al. (1985) *Tetrahedron Lett* 26:647; and Sato et al. (1986) *J Chem Soc Perkin Trans* 1:1231), b-aminoalcohols (Gordon et al. (1985) *Biochem Biophys Res Commun*126:419; and Dann et al. (1986) *Biochem Biophys Res Commun* 134:71), diaminoketones (Natarajan et al. (1984) *Biochem Biophys Res Commun* 124:141), and methyleneamino-modifed (Roark et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988, p134). Also, see generally, Session III: Analytic and synthetic methods, in in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988)

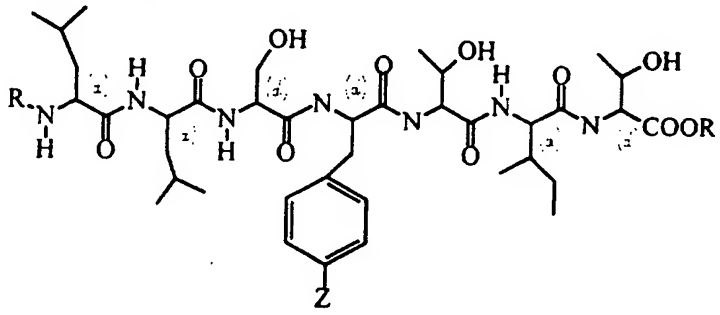
In an exemplary embodiment, the peptidomimetic can be derived as a retro-inverso analog of the peptide. To illustrate, the TITYSLL peptide can be generated as the retro-inverso analog:



Such retro-inverso analogs can be made according to the methods known in the art, such as that described by the Sisto et al. U.S. Patent 4,522,752. For example, the illustrated retro-inverso analog can be generated as follows. The geminal diamine corresponding to the N-terminal threonine is synthesized by treating a protected threonine analog with ammonia

under HOBT-DCC coupling conditions to yield the N-Boc amide, and then effecting a Hofmann-type rearrangement with I,I-bis-(trifluoroacetoxy)iodobenzene (TIB), as described in Radhakrishna et al. (1979) *J. Org. Chem.* 44:1746. The product amine salt is then coupled to a side-chain protected (e.g., as the benzyl ester) N-Fmoc D-Ile residue under standard conditions to yield the pseudodipeptide. The Fmoc (fluorenylmethoxycarbonyl) group is removed with piperidine in dimethylformamide, and the resulting amine is trimethylsilylated with bistrimethylsilylacetamide (BSA) before condensation with suitably alkylated, side-chain protected derivative of Meldrum's acid, as described in U.S. Patent 5,061,811 to Pinori et al., to yield the retro-inverso tripeptide analog. The pseudotripeptide is then coupled with with an L-phosphotyrosine analog under standard conditions to give the protected tetrapeptide analog. The protecting groups are removed to release the product, and the steps repeated to enlogate the tetrapeptide to the full length peptide. It will be understood that a mixed peptide, e.g. including some normal peptide linkages, can be generated. As a general guide, sites which are most susceptible to proteolysis are typically altered, with less susceptible amide linkages being optional for mimetic switching The final product, or intermediates thereof, can be purified by HPLC.

In another illustrative embodiment, the peptidomimetic can be derived as a retro-enatio analog of the peptide, such as the exemplary retro-enatio peptide analog derived for the illustrative TITYSSL peptide:

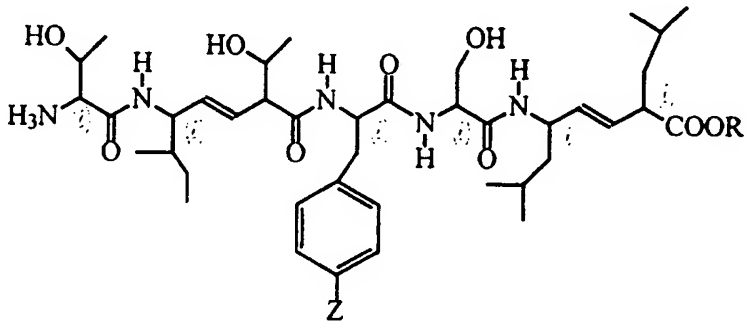


NH_3-(d) Leu-(d) Leu-(d) Ser-(d)Tyr-(d) Thr-(d) Ile-(d) Thr

Retro-enantio analogs such as this can be synthesized using D-enatiomers of tyrosine or phosphotyrosine analogs and commercially available D-amino acids and standard solid- or solution-phase peptide-synthesis techniques. For example, in a preferred solid-phase synthesis method, a suitably amino-protected (t-butyloxycarbonyl, Boc) D-threonine residue (or analog thereof) is covalently bound to a solid support such as chloromethyl resin. The resin is washed with dichloromethane (DCM), and the BOC protecting group removed by treatment with TFA in DCM. The resin is washed and neutralized, and the next Boc-protected D-amino acid (D-Ile) is introduced by coupling with diisopropylcarbodiimide. The

resin is again washed, and the cycle repeated for each of the remaining amino acids in turn (D-Thr, D-pTyr, etc). When synthesis of the protected retro-enantio peptide is complete, the protecting groups are removed and the peptide cleaved from the solid support by treatment with hydrofluoric acid/anisole/dimethyl sulfide/thioanisole. The final product is purified by HPLC to yield the pure retro-enantio analog.

In still another illustrative embodiment, trans-olefin derivatives can be made for the subject polypeptide. For example, an exemplary olefin analog is derived for the illustrative TITYSSL peptide:



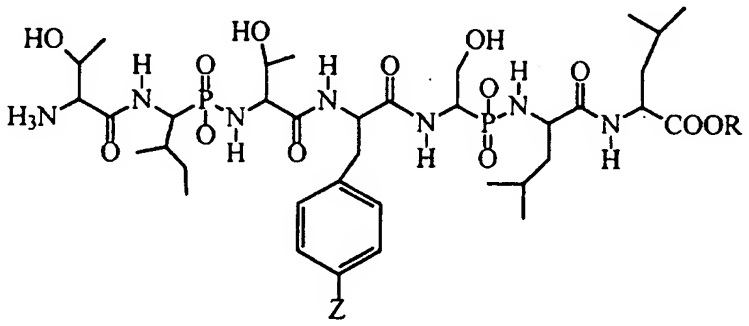
The trans olefin analog of a phosphotyroinse-containing peptide can be synthesized according to the method of Y.K. Shue et al. (1987) *Tetrahedron Letters* 28:3225. Referring to the illustrated example, Boc-amino L-Ile is converted to the corresponding α-amino aldehyde, which is treated with a vinylcuprate to yield a diastereomeric mixture of alcohols, which are carried on together. The allylic alcohol is acetylated with acetic anhydride in pyridine, and the olefin is cleaved with osmium tetroxide/sodium periodate to yield the aldehyde, which is condensed with the Wittig reagent derived from a protected threonine precursor, to yield the allylic acetate. The allylic acetate is selectively hydrolyzed with sodium carbonate in methanol, and the allylic alcohol is treated with triphenylphosphine and carbon tetrabromide to yield the allylic bromide. This compound is reduced with zinc in acetic acid to give the transposed trans olefin as a mixture of diastereomers at the newly-formed center. The diastereomers are separated and the pseudodipeptide is obtained by selective transfer hydrogenolysis to unveil the free carboxylic acid.

The pseudodipeptide is then coupled at the C-terminus with a suitably protected phosphotyrosine residue, and at the N-terminus with the protected threonine residue, by standard techniques, to yield the protected tetrapeptide isostere. The terapeptide is then further condensed with the olefinic tripeptide analog derived by similar means for Ser-Leu-Leu. The protecting groups are then removed with strong acid to yield the desired peptide analog, which can be further purified by HPLC.

Other pseudodipeptides can be made by the method set forth above merely by substitution of the appropriate starting Boc amino acid and Wittig reagent. Variations in the procedure may be necessary according to the nature of the reagents used, but any such variations will be purely routine and will be obvious to one of skill in the art.

It is further possible couple the pseudodipeptides synthesized by the above method to other pseudodipeptides, to make peptide analogs with several olefinic functionalities in place of amide functionalities. For example, pseudodipeptides corresponding to pTyr-Ser, Thr-Ile, Ser-Leu, etc. could be made and then coupled together by standard techniques to yield an analog of the ITIM peptide which has alternating olefinic bonds between residues.

Still another class of peptidomimetic derivatives include the phosphonate derivatives, such as the partially phosphonate derivatived TITYSSL peptide:



The synthesis of such phosphonate derivatives can be adapted from known synthesis schemes. See, for example, Loots et al. in *Peptides: Chemistry and Biology*, (Escom Science Publishers, Leiden, 1988, p. 118); Petrillo et al. in *Peptides: Structure and Function* (Proceedings of the 9th American Peptide Symposium, Pierce Chemical Co. Rockland, IL, 1985).

As set out above, a core ITIM motif for PTP1C activation is given by the general formula (I/V)XYXX(L/V), preferably the tyrosine is phosphorylated. Accordingly, each of the exemplary peptidomimetics above can be derived to correspond to the hexameric ITYSSL core ITIM sequence.

In certain embodiments, the ITIM mimetope will be able to cross a lipid bilayer, such as a plasma membrane of a cell. Mimetopes capable of crossing a lipid bilayer can be organic molecules, in particular carbohydrate-based molecules. Also according to the present invention, a compound of the present invention can include concatomers, wherein one or more ITIM peptides are linked together. As described above, various peptidomimetic derivatives of the peptides can be generated.

This invention further contemplates a method of generating sets of combinatorial libraires of the subject ITIM motifs which is especially useful for identifying potential variant sequences (e.g. homologs) that are functional in binding to a regulatory target molecule. The purpose of screening such combinatorial libraries is to generate, for example, novel homologs which can act as either agonists or antagonist, or alternatively, possess novel activities all together. To illustrate, ITIM homologs can be engineered by the present method to provide more efficient binding to PTP1C. Thus, combinatorially-derived homologs can be generated which have a enhanced potency relative to ITIMs generated from naturally occurring proteins.

Likewise, mutagenesis can give rise to homologs which have intracellular half-lives dramatically different than the corresponding wild-type ITIM. For example, the altered peptide can be rendered either more stable or less stable to proteolytic degradation or other cellular process which result in destruction of, or otherwise inactivation of, the peptide. Such homologs can be utilized to alter the envelope of therapeutic expression by modulating the half-life of the peptide. For instance, a short half-life can give rise to more transient biological effects and can allow tighter control of peptide levels within the cell.

In a representative embodiment of this method, the amino acid sequences for a population of ITIM motifs are aligned, preferably to promote the highest homology possible. Such a population of variants can include, for example, FcγRIIB, Ly49, or NKIRp58 (see Figure 1) from one or more cell types or species. Amino acids which appear at each position of the aligned sequences are selected to create a degenerate set of combinatorial sequences. The presence or absence of amino acids from an aligned sequence of a particular variant is relative to a chosen consensus length of a reference sequence, which can be real or artificial. In order to maintain the highest homology in alignment of sequences, deletions in the sequence of a variant relative to the reference sequence can be represented by an amino acid space (-), while insertional mutations in the variant relative to the reference sequence can be disregarded and left out of the sequence of the variant when aligned.

To illustrate, as set out above inspection of ITIM sequences reveals a consensus sequence of (I/V)XYXX(L/V) (see Figure 1). Based on these alignments, combinatorial libraries can be generated from this portion of ITIM peptides, the members of which can be expressed in the absence of other portions of receptor proteins, as a part of a receptor protein in which other portions of the protein are static (e.g. an FcγR, NKIR, etc.) in which only residues corresponding to the ITIM core sequence are varied by combinatorial mutagenesis, or as part of an unrelated fusion protein, e.g., as part of a phage coat protein or thioredoxin protein. In an exemplary embodiment, a library of ITIM homologs can be generated based on

the receptor ITIM sequences of Figure 1 so as to have an amino acid sequence represented by the degenerate formula:

(Ile/Val)-Xaa(1)-Tyr-Xaa(2)-Xaa(3)-(Leu/Val)

wherein each of Xaa(1)-Xaa(3) are selected from one of the amino acid residues of the same position in Figure 1, e.g., Xaa(1) can be a Val, Ile, Leu, Ser, Thr, Asp or His; Xaa(2) can be an Ala, Val, Ser or Thr; and Xaa(3) can be a Leu, Ile, Thr, Net, Gln, Glu or Asp.

Further expansion of the combinatorial library can be made, for example, by including amino acids which would represent conservative mutations at one or more of the degenerate positions. Inclusion of such conservative mutations can give rise to a library of potential cell-cycle regulatory sequences represented by the above formula, but wherein Xaa(1) can be a Gly, Ala, Val, Ile, Leu, Ser, Thr, Cys, Glu, Asp, Lys, Arg or His; Xaa(2) can be a Gly, Ala, Val, Ile, Leu, Ser, Cys or Thr; and Xaa(3) can be a Gly, Ala, Val, Ile, Leu, Ser, Cys, Thr, Met, Asn, Gln, Glu or Asp. Alternatively, amino acid replacement at degenerate positions can be based on steric criteria, e.g. isosteric replacement, without regard for polarity or charge of amino acid sidechains. Similarly, completely random mutagenesis of one or more of the variant positions (Xaa) can be carried out, e.g., each of Xaa(1)-(3) can be any of the 20 amino acids (or other analogs thereof).

In one embodiment the ITIM library can be derived by combinatorial chemistry, such as by techniques which are available in the art for generating combinatorial libraries of small organic/peptide libraries. See, for example, Blondelle et al. (1995) *Trends Anal. Chem.* 14:83; the Affymax U.S. Patents 5,359,115 and 5,362,899; the Ellman U.S. Patent 5,288,514; the Still et al. PCT publication WO 94/08051; Chen et al. (1994) *JACS* 116:2661; Kerr et al. (1993) *JACS* 115:252; PCT publications WO92/10092, WO93/09668 and WO91/07087; and the Lerner et al. PCT publication WO93/20242).

In a preferred embodiment, the combinatorial peptide library is produced by way of a degenerate library of genes encoding a library of polypeptides which each include at least a portion of potential ITIM sequences. For instance, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential ITIM nucleotide sequences are expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g. for phage display) containing the set of ITIM peptide sequences therein.

There are many ways by which the gene library of potential ITIM homologs can be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be carried out in an automatic DNA synthesizer, and the synthetic genes

then be ligated into an appropriate gene for expression. The purpose of a degenerate set of genes is to provide, in one mixture, all of the sequences encoding the desired set of potential ITIM sequences. The synthesis of degenerate oligonucleotides is well known in the art (see for example, Narang, SA (1983) *Tetrahedron* 39:3; Itakura et al. (1981) *Recombinant DNA, Proc 3rd Cleveland Sympos. Macromolecules*, ed. AG Walton, Amsterdam: Elsevier pp273-289; Itakura et al. (1984) *Annu. Rev. Biochem.* 53:323; Itakura et al. (1984) *Science* 198:1056; Ike et al. (1983) *Nucleic Acid Res.* 11:477. Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al. (1990) *Science* 249:386-390; Roberts et al. (1992) *PNAS* 89:2429-2433; Devlin et al. (1990) *Science* 249: 404-406; Cwirla et al. (1990) *PNAS* 87: 6378-6382; as well as U.S. Patents Nos. 5,223,409, 5,198,346, and 5,096,815).

A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of ITIM sequences. The most widely used techniques for screening large gene libraries typically comprises cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Such illustrative assays are amenable to high through-put analysis as necessary to screen large numbers of degenerate sequences created by combinatorial mutagenesis techniques.

In an illustrative embodiment of a screening assay, the ITIM gene library can be expressed as a fusion protein on the surface of a viral particle. For instance, in the filamentous phage system, foreign peptide sequences can be expressed on the surface of infectious phage, thereby conferring two significant benefits. First, since these phage can be applied to affinity matrices at very high concentrations, a large number of phage can be screened at one time. Second, since each infectious phage displays the combinatorial gene product on its surface, if a particular phage is recovered from an affinity matrix in low yield, the phage can be amplified by another round of infection. The group of almost identical *E.coli* filamentous phages M13, fd, and f1 are most often used in phage display libraries, as either of the phage gIII or gVIII coat proteins can be used to generate fusion proteins without disrupting the ultimate packaging of the viral particle (Ladner et al. PCT publication WO 90/02909; Garrard et al., PCT publication WO 92/09690; Marks et al. (1992) *J. Biol. Chem.* 267:16007-16010; Griffths et al. (1993) *EMBO J* 12:725-734; Clackson et al. (1991) *Nature* 352:624-628; and Barbas et al. (1992) *PNAS* 89:4457-4461).

For example, the recombinant phage antibody system (RPAS, Pharmacia Catalog number 27-9400-01) can be easily modified for use in expressing and screening ITIM motif combinatorial libraries of the present invention. For instance, the pCANTAB 5 phagemid of the RPAS kit contains the gene which encodes the phage gIII coat protein. The ITIM combinatorial gene library can be cloned into the phagemid adjacent to the gIII signal sequence such that it will be expressed as a gIII fusion protein. After ligation, the phagemid is used to transform competent *E. coli* TG1 cells. Transformed cells are subsequently infected with M13KO7 helper phage to rescue the phagemid and its candidate ITIM gene insert. The resulting recombinant phage contain phagemid DNA encoding a specific candidate ITIM peptide, and display one or more copies of the corresponding fusion coat protein. The phage-displayed candidate proteins which are capable of, for example, binding a PTP1C target, are selected or enriched by panning. For instance, the phage library can be panned on glutathione immobilized PTP1C-GST fusion proteins, and unbound phage washed away from the cells. The bound phage is then isolated, and if the recombinant phage express at least one copy of the wild type gIII coat protein, they will retain their ability to infect *E. coli*. Thus, successive rounds of reinfection of *E. coli*, and panning will greatly enrich for ITIM homologs which can then be screened for further biological activities in order to determine biological effects. Subsequent selection, e.g. of a reduced set of variants from the library, may then be based upon more meaningful criteria rather than simple PTP1C-binding ability. For instance, intracellular half-life or selectivity can become selection criteria in secondary screens.

Another aspect of the present invention includes a formulation comprising a regulatory reagent of the present invention. As used herein, the term "a" can refer to at least one (i.e., one or more). A formulation of the present invention can include a regulatory reagent that is capable of altering the activity of a molecule including PTP1C, PTP1D, ITIM-p160 and ITIM-p70 in a cell in such a manner that the activity of said molecule is altered resulting in regulation of hematopoietic cell function. Preferably, a formulation of the present invention comprises one or more peptides having the amino acid sequence TAENTITYSELLKH, GAENTITYSLLMH, or mimetopes thereof. More preferably, the tyrosine residue is phosphorylated. In more preferred embodiments an ITIM is represented by one of: VTYAQL, IVYTEL, VTYTQL, IVYTEL, VTYAQL, ILYTEL, VTYSMV, VTYTTL, VTYSTV, VIYSDL, or ITYAEL, and preferably the tyrosine residue is phosphorylated. Preferably an ITIM comprises one of ITYSLL or IXYXXL, and more preferably the tyrosine residue iw phosphorylated. In particularly preferred embodiments the formulation comprises (I/V)XYXX(L/V), and preferably the tyrosine residue is phosphorylated.

For example, the present invention provides pharmaceutically acceptable compositions which comprise a therapeutically-effective amount of one or more of the ITIM mimetics described herein, formulated together with one or more pharmaceutically acceptable carriers (additives) and/or diluents. As described in detail below, the pharmaceutical compositions of the present invention may be specially formulated for administration in solid or liquid form, including those adapted for the following: (1) oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, boluses, powders, granules, pastes for application to the tongue; (2) parenteral administration, for example, by subcutaneous, intramuscular or intravenous injection as, for example, a sterile solution or suspension; (3) topical application, for example, as a cream, ointment or spray applied to the skin; (4) intravaginally or intravectally, for example, as a pessary, cream or foam; or (5) as an aerosol or micronized powder spray, such as for inhalation.

The phrase "therapeutically-effective amount" as used herein means that amount of a compound, material, or composition comprising an ITIM peptide or peptidomimetic of the present invention which is effective for producing some desired therapeutic effect by inhibiting or, alternatively, potentiating an intracellular signalling pathway in at least a sub-population of cells in an animal and thereby altering the biological consequences of that pathway in the treated cells, at a reasonable benefit/risk ratio applicable to any medical treatment.

In one embodiment, an effective amount of a regulatory reagent to contact a cell with includes an amount capable of regulating a signal transduction pathway in a B cell. A preferred effective amount of a regulatory reagent comprises an amount that is capable of reducing B cell receptor activation in a cell contacted with the reagent. A more preferred effective amount of a regulatory reagent comprises an amount that is capable of dephosphorylating (i.e., reducing the extent of phosphorylation) of one or more signal transduction molecules functionally associated with a B cell receptor (i.e., a molecule, the activity of which is stimulated or inhibited by antigen binding to the B cell receptor). An even more preferred effective amount of a regulatory reagent comprises an amount that is capable of inducing dephosphorylation of, CD19, Fyn, Lyn, Syk, PI-3 kinase, Igα, Igβ, FcεRI β, FcεRI γ, FcγRIIA, CD3γ, CD3ε, TCR-zeta, Shc and/or PLC γ. The amount of dephosphorylation of a signal transduction molecule can be measured using methods known to those of skill in the art. In particular, dephosphorylation assays can be performed using the methods described in detail in Example 5.

In another embodiment of the present invention, an effective amount of a regulatory reagent to contact a cell with includes an amount capable of reducing the extent of intracellular calcium mobilization resulting from B cell receptor cross-linking, such reduction

compared with the extent of intracellular calcium mobilization resulting from B cell receptor cross-linking in the absence of the reagent. Preferably, an effective amount of a regulatory reagent comprises an amount capable of reducing calcium mobilization by about 2-fold, more preferably by about 3-fold and even more preferably by about 4-fold, when compared with the extent of intracellular calcium mobilization resulting from BCR cross-linking in the absence of the reagent. The amount of calcium mobilization in response to BCR cross-linking can be measured using methods known to those of skill in the art. In particular, the calcium mobilization can be measured using the general methods described in Example 6.

In another embodiment of the present invention, an effective amount of a regulatory reagent to contact a cell with includes an amount capable of reducing the amount of histamine produced by a cell. Preferably, an effective amount of a regulatory reagent comprises an amount capable of reducing the level of histamine production by a cell by about 2-fold. Similarly and in a different embodiment, an effective amount of a regulatory reagent for use with the method of the present invention comprises an amount that alters the level of IL-5 production by a cell preferably by about 2-fold, more preferably by about 3-fold and even more preferably by about 4-fold. Also similarly and in a different embodiment, an effective amount of a regulatory reagent for use with the method of the present invention comprises an amount that alters the level of IL-8 production by a cell preferably by about 2-fold, more preferably by about 3-fold and even more preferably by about 4-fold. The concentration of histamine, IL-5 and IL-8 produced by a cell can be measured using methods known to those of skill in the art. In particular, the concentration of IL-5 or IL-8 can be measured by, for example, using antibodies that specifically bind to Il-5 or IL-8, respectively, in an enzyme-linked immunoassay or a radioimmunoassay.

The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

The phrase "pharmaceutically-acceptable carrier" as used herein means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject peptide and peptidomimetic agents from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato

starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical formulations.

As set out above, certain embodiments of the subject ITIM mimetics may contain a basic functional group, such as amino or alkylamino, and are, thus, capable of forming pharmaceutically-acceptable salts with pharmaceutically-acceptable acids. The term "pharmaceutically-acceptable salts" in this respect, refers to the relatively non-toxic, inorganic and organic acid addition salts of peptides or peptidomimetics of the present invention. These salts can be prepared *in situ* during the final isolation and purification of the compounds of the invention, or by separately reacting a purified peptide or peptidomimetic in its free base form with a suitable organic or inorganic acid, and isolating the salt thus formed. Representative salts include the hydrobromide, hydrochloride, sulfate, bisulfate, phosphate, nitrate, acetate, valerate, oleate, palmitate, stearate, laurate, benzoate, lactate, phosphate, tosylate, citrate, maleate, fumarate, succinate, tartrate, napthylate, mesylate, glucoheptonate, lactobionate, and laurylsulphonate salts and the like. (See, for example, Berge et al. (1977) "Pharmaceutical Salts", *J. Pharm. Sci.* 66:1-19).

In other cases, the compounds of the present invention may contain one or more acidic functional groups and, thus, are capable of forming pharmaceutically-acceptable salts with pharmaceutically-acceptable bases. The term "pharmaceutically-acceptable salts" in these instances refers to the relatively non-toxic, inorganic and organic base addition salts of peptides and peptidomimetics of the present invention. These salts can likewise be prepared *in situ* during the final isolation and purification of the peptides or peptidomimetics, or by separately reacting the purified compound in its free acid form with a suitable base, such as the hydroxide, carbonate or bicarbonate of a pharmaceutically-acceptable metal cation, with ammonia, or with a pharmaceutically-acceptable organic primary, secondary or tertiary amine. Representative alkali or alkaline earth salts include the lithium, sodium, potassium, calcium, magnesium, and aluminum salts and the like. Representative organic amines useful for the formation of base addition salts include ethylamine, diethylamine, ethylenediamine, ethanolamine, diethanolamine, piperazine and the like. (See, for example, Berge et al., *supra*)

Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

Examples of pharmaceutically-acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

Formulations of the present invention include those suitable for oral, nasal, topical (including buccal and sublingual), rectal, vaginal, aerosol, and/or parenteral administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the host being treated, the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the peptide or peptidomimetic which produces a therapeutic effect. Generally, out of one hundred per cent, this amount will range from about 1 per cent to about ninety-nine percent of active ingredient, preferably from about 5 per cent to about 70 per cent, most preferably from about 10 per cent to about 30 per cent.

Methods of preparing these formulations or compositions include the step of bringing into association a compound of the present invention with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association a peptide or peptidomimetic of the present invention with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

Formulations of the invention suitable for oral administration may be in the form of capsules, cachets, pills, tablets, lozenges (using a flavored basis, usually sucrose and acacia or tragacanth), powders, granules, or as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil-in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia) and/or as mouth washes and the like, each containing a predetermined amount of a compound of the present invention as an active ingredient. A peptide or peptidomimetic of the present invention may also be administered as a bolus, electuary or paste.

In solid dosage forms of the invention for oral administration (capsules, tablets, pills, dragees, powders, granules and the like), the active ingredient is mixed with one or more pharmaceutically-acceptable carriers, such as sodium citrate or dicalcium phosphate, and/or any of the following: (1) fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and/or silicic acid; (2) binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose and/or acacia; (3) humectants, such as glycerol; (4) disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; (5) solution retarding agents, such as paraffin; (6) absorption accelerators, such as quaternary ammonium compounds; (7) wetting agents, such as, for example, cetyl alcohol and glycerol monostearate; (8) absorbents, such as kaolin and bentonite clay; (9) lubricants, such a talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof; and (10) coloring agents. In the case of capsules, tablets and pills, the pharmaceutical compositions may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared using binder (for example, gelatin or hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (for example, sodium starch glycolate or cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered peptide or peptidomimetic moistened with an inert liquid diluent.

The tablets, and other solid dosage forms of the pharmaceutical compositions of the present invention, such as dragees, capsules, pills and granules, may optionally be scored or prepared with coatings and shells, such as enteric coatings and other coatings well known in the pharmaceutical-formulating art. They may also be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile, other polymer matrices, liposomes and/or microspheres. They may be sterilized by, for example, filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved in sterile water, or some other sterile injectable medium immediately before use. These compositions may also optionally contain opacifying agents and may be of a composition that they release the active ingredient(s) only, or preferentially, in a certain portion of the gastrointestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric

substances and waxes. The active ingredient can also be in micro-encapsulated form, if appropriate, with one or more of the above-described excipients.

Liquid dosage forms for oral administration of the compounds of the invention include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. In addition to the active ingredient, the liquid dosage forms may contain inert diluents commonly used in the art, such as, for example, water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming and preservative agents.

Suspensions, in addition to the active peptides or peptidomimetics, may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

Formulations of the pharmaceutical compositions of the invention for rectal or vaginal administration may be presented as a suppository, which may be prepared by mixing one or more compounds of the invention with one or more suitable nonirritating excipients or carriers comprising, for example, cocoa butter, polyethylene glycol, a suppository wax or a salicylate, and which is solid at room temperature, but liquid at body temperature and, therefore, will melt in the rectum or vaginal cavity and release the active peptidomimetic.

Formulations of the present invention which are suitable for vaginal administration also include pessaries, tampons, creams, gels, pastes, foams or spray formulations containing such carriers as are known in the art to be appropriate.

Dosage forms for the topical or transdermal administration of a peptide or peptidomimetic of this invention include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active compound may be mixed under sterile conditions with a pharmaceutically-acceptable carrier, and with any preservatives, buffers, or propellants which may be required.

The ointments, pastes, creams and gels may contain, in addition to an active peptide or peptidomimetic of this invention, excipients, such as animal and vegetable fats, oils,

waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

Powders and sprays can contain, in addition to a compound of this invention, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

Transdermal patches have the added advantage of providing controlled delivery of a compound of the present invention to the body. Such dosage forms can be made by dissolving or dispersing the peptidomimetic in the proper medium. Absorption enhancers can also be used to increase the flux of the peptidomimetic across the skin. The rate of such flux can be controlled by either providing a rate controlling membrane or dispersing the peptidomimetic in a polymer matrix or gel.

Opthalmic formulations, eye ointments, powders, solutions and the like, are also contemplated as being within the scope of this invention.

Pharmaceutical compositions of this invention suitable for parenteral administration comprise one or more peptides or peptidomimetics of the invention in combination with one or more pharmaceutically-acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents.

Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition,

prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

In some cases, in order to prolong the effect of a drug, it is desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material having poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally-administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

Injectable depot forms are made by forming microencapsule matrices of the subject peptides or peptidomimetics in biodegradable polymers such as polylactide-polyglycolide. Depending on the ratio of drug to polymer, and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissue.

When the compounds of the present invention are administered as pharmaceuticals, to humans and animals, they can be given per se or as a pharmaceutical composition containing, for example, 0.1 to 99.5% (more preferably, 0.5 to 90%) of active ingredient in combination with a pharmaceutically acceptable carrier.

The preparations of the present invention may be given orally, parenterally, topically, or rectally. They are of course given by forms suitable for each administration route. For example, they are administered in tablets or capsule form, by injection, inhalation, eye lotion, ointment, suppository, etc. administration by injection, infusion or inhilation; topical by lotion or ointment; and rectal by suppositories. Oral administration is preferred.

The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticulare, subcapsular, subarachnoid, intraspinal and intrasternal injection and infusion.

The phrases "systemic administration," "administered systemically," "peripheral administration" and "administered peripherally" as used herein mean the administration of a compound, drug or other material other than directly into the central nervous system, such

that it enters the patient's system and, thus, is subject to metabolism and other like processes, for example, subcutaneous administration.

These peptides and peptidomimetics may be administered to humans and other animals for therapy by any suitable route of administration, including orally, nasally, as by, for example, a spray, rectally, intravaginally, parenterally, intracisternally and topically, as by powders, ointments or drops, including buccally and sublingually.

Regardless of the route of administration selected, the compounds of the present invention, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present invention, are formulated into pharmaceutically-acceptable dosage forms by conventional methods known to those of skill in the art.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of this invention may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient.

The selected dosage level will depend upon a variety of factors including the activity of the particular peptide or peptidomimetic of the present invention employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular peptidomimetic employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the compounds of the invention employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved.

In general, a suitable daily dose of a peptide or peptidomimetic of the invention will be that amount of the compound which is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above. Generally, intravenous, intracerebroventricular and subcutaneous doses of the compounds of this invention for a patient, when used for the indicated analgesic effects, will range from about 0.0001 to about 100 mg per kilogram of body weight per day.

If desired, the effective daily dose of the active peptide or peptidomimetic may be administered as two, three, four, five, six or more sub-doses administered separately at appropriate intervals throughout the day, optionally, in unit dosage forms.

While it is possible for a compound of the present invention to be administered alone, it is preferable to administer the peptide or peptidomimetic as a pharmaceutical formulation (composition).

Yet another aspect of the present invention relates to an isolated nucleic acid molecule that encodes a peptidyl form of the compounds of the present invention as herein disclosed. According to the present invention, references to nucleic acids also refer to nucleic acid molecules. In accordance with the present invention, an isolated nucleic acid molecule is a nucleic acid molecule that has been removed from its natural milieu (i.e., that has been subject to human manipulation). As such, "isolated" does not necessarily reflect the extent to which the nucleic acid molecule has be purified. An isolated nucleic acid molecule can include DNA, RNA, or hybrid or derivatives of either DNA or RNA. Nucleic acid molecules of the present invention can include a regulatory region that controls expression of the nucleic acid molecule (e.g., transcription or translation control regions), full-length or partial coding regions, and combinations thereof. Any portion of a nucleic acid molecule the present invention can be produced by (1) isolating the molecule from its natural milieu; (2) using recombinant DNA technology (e.g., PCR Amplification, cloning); or (3) using chemical synthesis methods.

A nucleic acid molecule of the present invention can include functional equivalents of natural nucleic acid molecules encoding a compound of the present invention including, but not limited to, natural allelic variants and modified nucleic acid molecules in which nucleotides have been inserted, deleted, substituted, and/or inverted in such a manner that such modifications do not substantially interfere with the nucleic acid molecules' ability to encode a compound of the present invention capable of regulating signal transduction. Preferred functional equivalents include nucleic acid sequences that are capable of hybridizing under stringent conditions, to: at least a portion of a nucleic acid molecule encoding an ITIM peptide derived from, e.g., an FcγR, CD22, NKIRp58 or Ly49 protein. Stringent hybridization conditions are described in Sambrook et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Labs Press, 1989, which is incorporated herein by reference in its entirety).

As guidance in determining what particular modifications can be made to any particular nucleic acid molecule, one of skill in the art should consider several factors that, without the need for undue experimentation, permit a skilled artisan to appreciate workable embodiments of the present invention. For example, such factors include modifications to nucleic acid molecules performed in a manner so as to maintain particular functional regions of the encoded compound of the present invention including: an ITIM motif capable of being bound by PTP1C, PTP1D, ITIM-p160 and/or ITIM-p70, capable of regulating the activity of

such proteins and a functional phosphatase domain. Functional tests of these various characteristics (e.g., binding studies, phosphatase assays, lipid phosphorylation assays) allows one of skill in the art to determine what modifications to the nucleic acid molecules would be appropriate and which would not. Functional tests of these various characteristics (e.g., binding studies, kinase assays, lipid phosphorylation assays) allows one of skill in the art to determine what modifications to the nucleic acid molecules would be appropriate and which would not.

In one embodiment an ITIM nucleic acid of the present invention encodes a polypeptide including an ITIM peptide sequence represented in the consensus polypeptide (I/V)XYXX(L/V). A preferred ITIM nucleic acid includes a coding sequence for an ITIM motif comprising an amino acid sequence ITYSLL or IXYXXL such as may be found in the peptides TAENTITYSELLKH, GAENTITYSLLMH. Other ITIM nucleic acid molecules encode ITIM peptides including such ITIM peptide sequences as VTYAQL, IVYTEL,VTYTQL, IVYTEL, VTYAQL, ILYTEL, VTYSMV, VTYTTL, VTYSTV, VIYSDL, or ITYAEL.

In another one embodiment, an isolated nucleic acid molecule of the present invention includes a nucleic acid sequence that encodes PTP1C, PTP1D, ITIM-p160 and/or ITIM-p70, as disclosed herein. A PTP1C nucleic acid molecule can include a nucleic acid sequence encoding at least a portion of a PTP1C protein having an amino acid sequence as disclosed in (Shen et al., Nature 352:736-739, 1991; Matthews et al., Mol. Cell. Biol. 12:2396-2405, 1992; and Plutzky et al., Proc. Natl. Acad. Sci. USA 89:1123-1127, 1992), or a mimetope thereof. In preferred embodiments, the nucleic acid encodes an ITIM-binding SH2 domain from PTP1C.

At least a portion of a nucleic acid molecule encoding an ITIM peptide can be covalently associated (using standard recombinant DNA methods) to any other sequence that codes for at least a portion of a distinct component (e.g., control element) to produce an ITIM peptide of the present invention. The sequences can be attached in such a manner so that the sequences are transcribed in-frame, thereby producing a functional ITIM peptide capable of regulating the activity of an SH2 containing tyrosine phosphatase.

In addition, the nucleic acid encoding the subject peptides can be used to generate a chimeric gene encoding a fusion protein. For example, a nucleic acid sequence can be added that encodes a signal sequence, e.g., a sequence which directs secretion of the recombinantly produced ITIM peptide from the host cell. Nucleic acid sequences encoding a signal sequence are covalently associated (by base pair linkage) to the 5' end (amino terminal end) of a nucleic acid molecule. Useful signal sequences are known in the art (c.f., Achstetter et al. (1992) *Gene* 110:25). Preferred signal or leader segments are segments naturally associated

with a FcγR, CD22, NKIRp58, or Ly49. Preferred transmembrane segments include segments that are naturally associated with a FcγR, CD22, NKIRp58, or Ly49.

To obtain membrane-bound forms of the ITIM peptides, chimeric nucleic acids sequences can be used which provide the peptide with at least one transmembrane segment (including stop transfer sequences) capable of anchoring the peptide to a lipid-containing target molecule or membrane. A nucleic acid sequence encoding a transmembrane segment is covalently associated (by base pair linkage) to one of either the 5' or 3' end of a nucleic acid molecule encoding an ITIM peptide of the present invention. A chimeric nucleic acid molecule encoding a fusion polypeptide comprising the peptide of the present invention with a transmembrane domain at its N-terminus can be used to provide a membrane anchored form of the ITIM peptides in which the peptide of interest is presented at the cytoplasmic face of the cell membrane. Alternatively, a chimeric gene can be constructed which provides a nucleic acid sequence encoding a transmembrane sequence ligated to the 3' end of a nucleic acid sequence encoding an ITIM so as to provide that peptide at the extracellular face of the cell membrane. Moreover, it is clear that the choice of signal peptides and/or transmembrane sequences and their orientation relative to the ITIM peptide sequences in the chimeric gene can be used to localize the subject peptides in various intracellular compartments. In addition, it will be apparent that other membrane anchoring motifs can be employed, such as to direct prenylation of a chimeric peptide (e.g., using a farnesyltransferase or geranylgeranyl transferase recognition sequence), or to direct attachment of phosphatidylinositol to the peptide.

In addition to utilizing fusion proteins to direct localization of the subject peptides, it is widely appreciated that fusion proteins can enhance production/purification of peptides. For example, inclusion of a fusion segment as part of a compound of the present invention can enhance the stability of the compound during production, storage and/or use. Furthermore, a fusion segment can function as a tool to simplify purification of a compound of the present invention, such as to enable purification of the resultant fusion protein using affinity chromatography. For example, an ITIM peptide can be generated as a glutathione-S-transferase (GST) fusion protein. Such GST-fusion proteins can enable easy purification of the subject peptides, as for example by the use of glutathione-derivatized matrices (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al. (N.Y.: John Wiley & Sons, 1991)). Addition of a proteolytic cleavage site, such as a Factor X cleavage site, between the GST moiety and subject peptide permits release of the subject peptide. In another embodiment, a fusion gene coding for a purification leader sequence, such as a poly-(His)/enterokinase cleavage site sequence, can be used as the signal sequence at N-terminus the peptide in order to permit purification of the poly(His)-peptide by affinity

chromatography using a Ni^{2+} metal resin. The purification leader sequence can then be subsequently removed by treatment with enterokinase (e.g., see Hochuli et al. (1987) *J. Chromatography* 411:177; and Janknecht et al. *PNAS* 88:8972).

Techniques for making fusion genes are well known. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. Alternatively, the chimeric gene can be synthesized by conventional techniques including automated DNA synthesizers. In another method, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed to generate a chimeric gene sequence (see, for example, Current Protocols in Molecular Biology, Eds. Ausubel et al. John Wiley & Sons: 1992).

It may be necessary in some instances to introduce an unstructured polypeptide linker region between the ITIM peptide portion of the fusion protein and other fragments. This linker can facilitate enhanced flexibility of the fusion protein allowing the ITIM peptides to freely interact with target molecule, reduce steric hindrance between the two fragments, as well as allow appropriate folding of each fragment to occur. The linker can be of natural origin, such as a sequence determined to exist in random coil between two domains of a protein. Alternatively, the linker can be of synthetic origin. For instance, the sequence $(Gly_4Ser)_3$ can be used as a synthetic unstructured linker. Linkers of this type are described in Huston et al. (1988) PNAS 85:4879; and U.S. Patent No. 5,091,513.

The present invention also includes a recombinant molecule comprising a nucleic acid molecule encoding a compound of the present invention operatively linked to a vector capable of being expressed in a host cell. As used herein, "operatively linked" refers to insertion of a nucleic acid sequence into an expression vector in such a manner that the sequence is capable of being expressed when transformed into a cell. As used herein, an "expression vector" is an RNA or DNA vector capable of transforming a host cell and effecting expression of an appropriate nucleic acid molecule, preferably replicating within the host cell. An expression vector can be either prokaryotic or eukaryotic, and typically is a virus or a plasmid.

Construction of desired expression vectors can be performed by methods known to those skilled in the art and expression can be in eukaryotic or prokaryotic systems. Suitable prokaryotic systems are bacterial strains, including, but not limited to various strains of *E.coli* various strains of *bacilli* or various species of *Pseudomonas*. In prokaryotic systems,

plasmids are used that contain replication sites and control sequences derived from a species compatible with a host cell. Control sequences can include, but are not limited to promoters, operators, enhancers, ribosome binding sites, and Shine-Dalgarno sequences. Expression systems useful in eukaryotic host cells comprise promoters derived from appropriate eukaryotic genes. Useful mammalian promoters include early and late promoters from SV40 or other viral promoters such as those derived from baculovirus, polyoma virus, adenovirus, bovine papilloma virus or avian sarcoma virus. Expression vectors of the present invention include any vectors that function (i.e., direct gene expression) in recombinant cells of the present invention including bacterial, yeast, other fungal, insect, plant mammalian cells. An expression system can be constructed from any of the foregoing control elements operatively linked to the nucleic acid molecules of the present invention using methods known to those skilled in the art (see, for example, Sambrook, et al. *ibid.*).

Host cells of the present invention can be: cells naturally capable of producing a compound of the present invention; or cells that are capable of producing a compound of the present invention when transfected with a nucleic acid molecule encoding a compound of the present invention. Host cells of the present invention include, but are not limited to, bacterial, fungal, insect, plant and mammalian cells. More preferred host cells include *Escherichia*, *Bacillus*, *Saccharomyces*, SF9 and Drosophila.

A recombinant cell is preferably produced by transforming a host cell with one or more recombinant molecules, each comprising one or more nucleic acid molecules of the present invention operatively linked to an expression vector containing one or more transcription control sequences. A recombinant molecule of the present invention is a molecule that can include at least one of the nucleic acid molecules heretofore described, operatively linked to at least one of any transcription control sequence capable of effectively regulating expression of the nucleic acid molecule(s) in the cell to be transformed.

It may be appreciated by one skilled in the art that use of recombinant DNA technologies can improve expression of transformed nucleic acid molecules by manipulating, for example, the number of copies of the nucleic acid molecules within a host cell, the efficiency with which those nucleic acid molecules are transcribed, and the efficiency with which the resultant transcripts are translated, and the efficiency of post-translational modifications. Recombinant techniques useful for increasing the expression of nucleic acid molecules of the present invention include, but are not limited to, operatively linking nucleic acid molecules to high-copy number plasmids, integration of the nucleic acid molecules into one or more host cell chromosomes, addition of vector stability sequences to plasmids, substitutions or modifications of transcription control signals (e.g., promoters, operators, enhancers), substitutions or modifications of translational control signals, modification of

nucleic acid molecules of the present invention to correspond to the code on usage of the host cell, deletion of sequences that destabilize transcripts, and use of control signals that temporarily separate recombinant cell growth from recombinant enzyme production during fermentation. The activity of an expressed recombinant peptide of the present invention may be improved by fragmenting, modifying, or derivitizing nucleic acid molecules encoding such a peptide.

In accordance with the present invention, recombinant cells of the present invention can be used to produce a compound of the present invention by culturing such cells under conditions effective to produce such a compound, and recovering the compound. Effective conditions to produce a compound of the present invention include, but are not limited to, appropriate media, bioreactor, temperature, pH and oxygen conditions that permit peptide production. An appropriate medium refers to any medium in which a cell of the present invention, when cultured, is capable of producing a compound of the present invention. An effective medium is typically an aqueous medium comprising assimilable carbohydrate, nitrogen and phosphate sources, as well as appropriate salts, minerals, metals, and other nutrients, such as vitamins. The medium may comprise complex nutrients or may be a defined minimal medium. The medium may also contain chemical reagents which select for expression of particular recombinant molecules. Such reagents include, but are not limited to, neomycin, ampicillin, tetracycline, chloramphenicol and mycophenolic acid.

Depending on the vector and host system used for production, resultant compounds of the present invention may either remain within the recombinant cell; be secreted into the fermentation medium; be secreted into a space between two cellular membranes, such as the piroplasmic space in *E.coli*; or be retained on the outer surface of a cell or viral membrane. The phrase "recovering the compound" refers simply to collecting the whole fermentation medium containing the compound and need not imply additional steps of separation or purification. A compound of the present invention can be purified using a variety of standard protein purification techniques, such as, but not limited to, affinity chromatography, ion exchange chromatography, filtration, electrophoresis, hydrophobic interaction chromatography, gel filtration chromatography, reverse phase chromatography, concanavalin A chromatography, chromatafocusing, differential solubilization, and immunoprecipitation. A compound of the present invention is preferably retrieved in "substantially pure" form. As used herein, "substantially pure" refers to a purity that allows for the effective use of the compound as a therapeutic composition or in an assay system. A substantially pure compound of the present invention, for example, should be capable of regulating the activity of an SH2 domain containing tyrosine phosphatase in a cell without exhibiting substantial toxicity to such cell.

In addition to providing a source of recombinant peptides, the expression vector of the present application can also be used to generate a variety of gene therapy compositions. Accordingly, the expression constructs of the subject ITIM peptides may be administered in any biologically effective carrier, e.g. any formulation or composition capable of effectively transfecting cells *in vivo* with a recombinant gene. Approaches include insertion of the subject gene in viral vectors including recombinant retroviruses, adenovirus, adeno-associated virus, and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. Viral vectors can be used to transfect cells directly; plasmid DNA can be delivered with the help of, for example, cationic liposomes (lipofectin) or derivatized (e.g. antibody conjugated), polylysine conjugates, gramacidin S, artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or $CaPO_4$ precipitation carried out *in vivo*. It will be appreciated that because transduction of appropriate target cells represents the critical first step in gene therapy, choice of the particular gene delivery system will depend on such factors as the phenotype of the intended target and the route of administration, e.g. locally or systemically.

A preferred approach for *in vivo* introduction of nucleic acid encoding one of the subject proteins into a cell is by use of a viral vector containing nucleic acid, e.g. a cDNA, encoding the gene product. Infection of cells with a viral vector has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in the viral vector, are expressed efficiently in cells which have taken up viral vector nucleic acid.

Retrovirus vectors and adeno-associated virus vectors are generally understood to be the recombinant gene delivery system of choice for the transfer of exogenous genes *in vivo*, particularly into humans. These vectors provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host. A major prerequisite for the use of retroviruses is to ensure the safety of their use, particularly with regard to the possibility of the spread of wild-type virus in the cell population. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) *Blood* 76:271). Thus, recombinant retrovirus can be constructed in which part of the retroviral coding sequence (*gag, pol, env*) has been replaced by nucleic acid encoding one of the subject peptides, rendering the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo*

with such viruses can be found in *Current Protocols in Molecular Biology*, Ausubel, F.M. et al., (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include ψCrip, ψCre, ψ2 and ψAm. Retroviruses have been used to introduce a variety of genes into many different cell types, including neural cells, epithelial cells, endothelial cells, lymphocytes, myoblasts, hepatocytes, bone marrow cells, *in vitro* and/or *in vivo* (see for example Eglitis et al., (1985) *Science* 230:1395-1398; Danos and Mulligan, (1988) *Proc. Natl. Acad. Sci. USA* 85:6460-6464; Wilson et al., (1988) *Proc. Natl. Acad. Sci. USA* 85:3014-3018; Armentano et al., (1990) *Proc. Natl. Acad. Sci. USA* 87:6141-6145; Huber et al., (1991) *Proc. Natl. Acad. Sci. USA* 88:8039-8043; Ferry et al., (1991) *Proc. Natl. Acad. Sci. USA* 88:8377-8381; Chowdhury et al., (1991) *Science* 254:1802-1805; van Beusechem et al., (1992) *Proc. Natl. Acad. Sci. USA* 89:7640-7644; Kay et al., (1992) *Human Gene Therapy* 3:641-647; Dai et al., (1992) *Proc. Natl. Acad. Sci. USA* 89:10892-10895; Hwu et al., (1993) *J. Immunol.* 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573).

Furthermore, it has been shown that it is possible to limit the infection spectrum of retroviruses and consequently of retroviral-based vectors, by modifying the viral packaging proteins on the surface of the viral particle (see, for example PCT publications WO93/25234, WO94/06920, and WO94/11524). For instance, strategies for the modification of the infection spectrum of retroviral vectors include: coupling antibodies specific for cell surface antigens to the viral *env* protein (Roux et al., (1989) *Proc. Natl. Acad. Sci. USA* 86:9079-9083; Julan et al., (1992) *J. Gen Virol* 73:3251-3255; and Goud et al., (1983) *Virology* 163:251-254); or coupling cell surface ligands to the viral *env* proteins (Neda et al., (1991) *J. Biol. Chem.* 266:14143-14146). Coupling can be in the form of the chemical cross-linking with a protein or other variety (e.g. lactose to convert the *env* protein to an asialoglycoprotein), as well as by generating fusion proteins (e.g. single-chain antibody/*env* fusion proteins). This technique, while useful to limit or otherwise direct the infection to certain tissue types, and can also be used to convert an ecotropic vector in to an amphotropic vector.

Moreover, use of retroviral gene delivery can be further enhanced by the use of tissue- or cell-specific transcriptional regulatory sequences which control expression of the recombinant gene of the retroviral vector.

Another viral gene delivery system useful in the present invention utilizes adenovirus-derived vectors. The genome of an adenovirus can be manipulated such that it encodes a gene product of interest, but is inactivate in terms of its ability to replicate in a normal lytic viral life cycle (see, for example, Berkner et al., (1988) *BioTechniques* 6:616; Rosenfeld et al., (1991) *Science* 252:431-434; and Rosenfeld et al., (1992) *Cell* 68:143-155). Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses can be advantageous in certain circumstances in that they are not capable of infecting nondividing cells and can be used to infect a wide variety of cell types, including airway epithelium (Rosenfeld et al., (1992) cited *supra*), endothelial cells (Lemarchand et al., (1992) *Proc. Natl. Acad. Sci. USA* 89:6482-6486), hepatocytes (Herz and Gerard, (1993) *Proc. Natl. Acad. Sci. USA* 90:2812-2816) and muscle cells (Quantin et al., (1992) *Proc. Natl. Acad. Sci. USA* 89:2581-2584). Furthermore, the virus particle is relatively stable and amenable to purification and concentration, and as above, can be modified so as to affect the spectrum of infectivity. Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al., *supra*; Haj-Ahmand and Graham (1986) *J. Virol.* 57:267). Most replication-defective adenoviral vectors currently in use and therefore favored by the present invention are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80% of the adenoviral genetic material (see, e.g., Jones et al., (1979) *Cell* 16:683; Berkner et al., *supra*; and Graham et al., in *Methods in Molecular Biology*, E.J. Murray, Ed. (Humana, Clifton, NJ, 1991) vol. 7. pp. 109-127). Expression of the inserted ITIM gene can be under control of, for example, the E1A promoter, the major late promoter (MLP) and associated leader sequences, the E3 promoter, or exogenously added promoter sequences.

Yet another viral vector system useful for delivery of the subject peptide-encoding genes is the adeno-associated virus (AAV). Adeno-associated virus is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review, see Muzyczka et al., *Curr. Topics in Micro. and Immunol.* (1992) 158:97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte et al., (1992) *Am. J. Respir. Cell. Mol. Biol.* 7:349-356; Samulski et al., (1989) *J. Virol.* 63:3822-3828; and McLaughlin et al., (1989) *J. Virol.*

62:1963-1973). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin et al., (1985) *Mol. Cell. Biol.* 5:3251-3260 can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat et al., (1984) *Proc. Natl. Acad. Sci. USA* 81:6466-6470; Tratschin et al., (1985) *Mol. Cell. Biol.* 4:2072-2081; Wondisford et al., (1988) *Mol. Endocrinol.* 2:32-39; Tratschin et al., (1984) *J. Virol.* 51:611-619; and Flotte et al., (1993) *J. Biol. Chem.* 268:3781-3790).

Other viral vector systems that may have application in gene therapy have been derived from herpes virus, vaccinia virus, and several RNA viruses. In particular, herpes virus vectors may provide a unique strategy for persistence of the recombinant gene in cells of the central nervous system and ocular tissue (Pepose et al., (1994) *Invest Ophthalmol Vis Sci* 35:2662-2666)

In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed to cause expression of a recombinant ITIM peptide in the tissue of an animal. Most nonviral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. In preferred embodiments, non-viral gene delivery systems of the present invention rely on endocytic pathways for the uptake of the peptide-encoding gene by the targeted cell. Exemplary gene delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes.

In a representative embodiment, a gene encoding a ITIM peptide can be entrapped in liposomes bearing positive charges on their surface (e.g., lipofectins) and (optionally) which are tagged with antibodies against cell surface antigens of the target tissue (Mizuno et al., (1992) *No Shinkei Geka* 20:547-551; PCT publication WO91/06309; Japanese patent application 1047381; and European patent publication EP-A-43075). For example, lipofection of lymphoma cells can be carried out using liposomes tagged with monoclonal antibodies against lymphomaa-associated antigens. Liposome delivery is also a well known method for delivering small peptides and even proteins directly into cells and can be used to directly deliver ITIM peptides and mimetics.

In yet another illustrative embodiment, the gene delivery system comprises an antibody or cell surface ligand which is cross-linked with a gene binding agent such as poly-lysine (see, for example, PCT publications WO93/04701, WO92/22635, WO92/20316, WO92/19749, and WO92/06180). For example, the subject peptide gene constructs can be used to transfect hepatocytic cells *in vivo* using a soluble polynucleotide carrier comprising an asialoglycoprotein conjugated to a polycation, e.g. poly-lysine (see U.S. Patent

5,166,320). It will also be appreciated that effective delivery of the subject nucleic acid constructs via -mediated endocytosis can be improved using agents which enhance escape of the gene from the endosomal structures. For instance, whole adenovirus or fusogenic peptides of the influenza HA gene product can be used as part of the delivery system to induce efficient disruption of DNA-containing endosomes (Mulligan et al., (1993) *Science* 260-926; Wagner et al., (1992) *Proc. Natl. Acad. Sci. USA* 89:7934; and Christiano et al., (1993) *Proc. Natl. Acad. Sci. USA* 90:2122).

In clinical settings, the gene delivery systems can be introduced into a patient by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g. by intravenous injection, and specific transduction of the construct in the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see U.S. Patent 5,328,470) or by stereotactic injection (e.g. Chen et al., (1994) *Proc. Natl. Acad. Sci. USA* 91: 3054-3057).

Another aspect of the present invention relates to a variety of drug screening assays designed to identify compounds which can modulate ITIM-dependent signal transduction pathways. In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Assays which are performed in cell-free systems, such as may be derived with purified or semi-purified proteins, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound. Moreover, the effects of cellular toxicity and/or bioavailability of the test compound can be generally ignored in the *in vitro* system, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest, for example, in an alteration of binding affinity between various signal transduction proteins.

While the ITIM/TPT1C interaction is described below for illustrative purposed, it will be clear that other interactions described herein, such as ITIM/PTP1D, ITIM/p160, ITIM/p70, and PTP1C/CD19, are each potential therapeutic targets and accordingly may be cast in drug screening assays similar to those described below.

In an exemplary screening assay of the present invention, the compound of interest is contacted with an isolated and purified polypeptide which is ordinarily capable of binding to an ITIM polypeptide sequence. Thus, to a mixture of the compound and PTP1C polypeptide

is then added a composition containing an ITIM polypeptide. The ITIM may be in the form of a short peptide, or may be a larger soluble portion of a protein containing an ITIM sequence, such as the cytoplasmid comain of FcγRIIB1. Detection and quantification of ITIM/PTP1C complexes provides a means for determining the compound's efficacy at inhibiting (or potentiating) complex formation between the ITIM and PTP1C polypeptides. The efficacy of the compound can be assessed by generating dose response curves from data obtained using various concentrations of the test compound. Moreover, a control assay can also be performed to provide a baseline for comparison. In the control assay, isolated and purified ITIM is added to a composition containing the PTP1C protein, and the formation of ITIM/PTP1C complex is quantitated in the absence of the test compound. It will be understood that, in general, the order in which the reactants may be admixed can be varied, and can be admixed simultaneously.

Complex formation between the target PTP1C polypeptide and ITIM polypeptide may be detected by a variety of techniques. For instance, modulation of the formation of complexes can be quantitated using, for example, detectably labelled proteins such as radiolabelled (e.g. ^{32}P, ^{35}S, ^{14}C or ^{3}H), fluorescently labelled (e.g. FITC), or enzymatically labelled PTP1C or ITIM polypeptides, by immunoassay, or by chromatographic detection. The use of enzymatically labeled PTP1C will, of course, generally be used only when enzymatically inactive portions of the phosphatase are used, such as an isolated SH2 domain as that protein can possess a measurable intrinsic activity which can be detected as described herein.

Typically, it will be desirable to immobilize either the PTP1C or the ITIM polypeptide to facilitate separation of PTP1C/ITIM complexes from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Binding of ITIM to PTP1C, in the presence and absence of a candidate agent, can be accomplished in any vessel suitable for containing the reactants. Examples include microtitre plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows the protein to be bound to a matrix. For example, glutathione-S-transferase/PTP1C (GST/PTP1C) fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with the ITIM polypeptide, e.g. an ^{35}S-labeled ITIM polypeptide, and the test compound, and the mixture incubated under conditions conducive to complex formation, e.g. at physiological conditions for salt and pH, though slightly more stringent conditions may be desired, e.g., at 4°C in a buffer containing 0.6M NaCl or a detergent such as 0.1% Triton X-100. Following incubation, the beads are washed to remove any unbound ITIM polypeptide, and the matrix immobilized radiolabel determined

directly (e.g. beads placed in scintilant), or in the supernatant after the PTP1C/ITIM complexes are subsequently dissociated. Alternatively, the complexes can dissociated from the matrix, separated by SDS-PAGE, and the level of ITIM polypeptide found in the bead fraction quantitated from the gel using standard electrophoretic techniques such as described in the appended examples.

Other techniques for immobilizing proteins on matrices are also available for use in the subject assay. For instance, either of the PTP1C or ITIM proteins can be immobilized utilizing conjugation of biotin and streptavidin. For instance, biotinylated PTP1C molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with the PTP1C but which do not interfere with ITIM binding can be derivatized to the wells of the plate, and the PTP1C trapped in the wells by antibody conjugation. As above, preparations of an ITIM polypeptide and a test compound are incubated in the PTP1C-presenting wells of the plate, and the amount of PTP1C/ITIM complex trapped in the well can be quantitated. Exemplary methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with the ITIM polypeptide, or which are reactive with the PTP1C protein and compete for binding with the ITIM polypeptide; as well as enzyme-linked assays which rely on detecting an extrinsic enzymatic activity associated with the ITIM polypeptide. In the instance of the latter, the enzyme can be chemically conjugated or provided as a fusion protein with an ITIM polypeptide. To illustrate, the ITIM polypeptide can be chemically cross-linked or genetically fused with horseradish peroxidase, and the amount of ITIM polypeptide trapped in the complex can be assessed with a chromogenic substrate of the enzyme, e.g. 3,3'-diamino-benzadine terahydrochloride or 4-chloro-1-napthol. Likewise, a fusion protein comprising the ITIM polypeptide and glutathione-S-transferase can be provided, and complex formation quantitated by detecting the GST activity using 1-chloro-2,4-dinitrobenzene (Habig et al (1974) *J Biol Chem* 249:7130).

For processes which rely on immunodetection for quantitating one of the proteins trapped in the complex, antibodies against the protein, such as either anti-ITIM or anti-PTP1C antibodies, can be used. Alternatively, the protein to be detected in the complex can be "epitope tagged" in the form of a fusion protein which includes, in addition to the ITIM polypeptide or PTP1C sequence, a second polypeptide for which antibodies are readily available (e.g. from commercial sources). For instance, the GST fusion proteins described above can also be used for quantification of binding using antibodies against the GST moiety. Other useful epitope tags include myc-epitopes (e.g., see Ellison et al. (1991) *J Biol Chem*

266:21150-21157) which includes a 10-residue sequence from c-myc, as well as the pFLAG system (International Biotechnologies, Inc.) or the pEZZ-protein A system (Pharamacia, NJ).

In still another embodiment, the polypeptides of the present invention can be disposed as part of a thioredoxin chimeric protein and the ability of the exogenous peptide to direct binding of the thioredoxin to a target protein can be assessed in the presence and absence of a candidate agent. To illustrate, a modified version of the *E. coli* thioredoxin (trxA) protein can be used as a larger carrier protein (LaVallie et al., *Biotechnology* 11:187-193, 1993). The crystal structure of trxA (~12kD) shows that the protein is extremely compact but the active site region which includes glycine 33 and proline 34 forms a protruding loop. The peptide inserted in the protuding loop between Gly33-Pro34 will be displayed on the surface of trxA. For example, proteins expressing peptides inserted in this loop have been succesfully used for the production of antipeptide monoclonal antibodies. Such peptide loops can be digested by enteropeptidase when the inserted peptide carries a recognition site for the enzyme (DDDK). Moreover, the trxA carrier protein has other additional desirable features. First, overexpression of trxA in*S. cerevisiae* cells does not negatively affect their growth. Second, fusion of insoluble peptides to the trxA protein helps to keep these proteins soluble in *E. coli*. Third, the trxA fusion proteins localize to the inner periphery of the cytoplasmic membrane in *E. coli* and can easily be released by osmotic shock. Finally, an important practical consideration, the existence of a unique RsrII restriction site in the DNA encoding glycine 33 and proline 34 allows for easy insertion of peptides

In an exemplary embodiment, an ITIM polypeptide can be provided in the active site loop of thioredoxin, and the ability of that molecule to bind to, for example, PTP1C or *PTP1D* assessed. Moreover, such constructs can be used to generate combinatorial libraries of related ITIMs, and these libraries screened to identify those variants which retain the ability to bind to the target molecule.

The above thioredoxin chimeras, and similar constructs thereto, can be used in direct cell-free binding assays. Alternatively, they can be used to generate an interaction trap assay(ITS), utilizing such systems as described in U.S. Patent No. 5,283,317; Zervos et al. (1993) *Cell* 72:223-232; Madura et al. (1993) *J Biol Chem* 268:12046-12054; Bartel et al. (1993) *Biotechniques* 14:920-924; and Iwabuchi et al. (1993) *Oncogene* 8:1693-1696. As in the cell-free binding assays, the ITS can be employed for detecting agents which disrupt binding of the ITIM peptide to a target protein, e.g. PTP1C.

The interaction trap assay relies on reconstituting *in vivo* a functional transcriptional activator protein from two separate fusion proteins, one of which comprises the DNA-binding domain of a transcriptional activator fused to a thioredoxin chimera, such as a Thrioredoxin/ITIM chimera. The second fusion protein comprises a transcriptional activation

domain (e.g. able to initiate RNA polymerase transcription) fused to, for example, a PTP1C polypeptide, preferably a catalytically inactive mutant. When the ITIM and PTP1C domains of each fusion protein interact, the two domains of the transcriptional activator protein are brought into sufficient proximity as to cause transcription of a reporter gene. By detecting the level of transcription of the reporter, the ability of a test agent to inhibit (or potentiate) binding of PTP1C to ITIM can be evaluated.

In an illustrative embodiment, *Saccharomyces cerevisiae* YPB2 cells are transformed simultaneously with a plasmid encoding a GAL4db-Thioredoxin/ITIM fusion and with a plasmid encoding the GAL4ad domain fused to a regulatory domain of PTP1C. Moreover, the strain is transformed such that the GAL4-responsive promoter drives expression of a phenotypic marker. For example, the ability to grow in the absence of histidine can depends on the expression of the HIS3 gene. When the HIS3 gene is placed under the control of a GAL4-responsive promoter, relief of this auxotrophic phenotype indicates that a functional GAL4 activator has been reconstituted through the interaction of ITIM and the PTP1C protein. Thus, a test agent able to inhibit PTP1C interaction with ITIM will result in yeast cells unable to growth in the absence of histidine. Alternatively, the phenotypic marker (e.g. instead of the HIS3 gene) can be one which provides a negative selection (e.g., are cytotoxic) when expressed such that agents which disrupt ITIM/PTP1C interactions confer positive growth selection to the cells.

Moreover, it will be apparent that the assays above can be generated for differential screening with two different potential targets for binding of ITIM motifs in order to generate specificity.

In yet other embodiments, the assay assesses the effect of a test agent on the enzymatic activity of a transduction protein interactive with one of the subject ITIM peptides. In an exemplary embodiment, such kits can include: (1) a tyrosine phosphatase; (2) a compound of the present invention capable of stimulating the activity of the tyrosine phosphatase; and (3) a means for measuring the activity of the tyrosine phosphatase. It is within the skill of one in the art to modify a kit of the present invention to identify inhibitory compounds by, for example, adding to the kit a primary target molecule capable of being modified by the tyrosine phosphatase and a means for detecting such modification. The activity of a tyrosine phosphatase can be measured by, for example, measuring dephosphorylation or modification of a primary target molecule, or a phosphorylated fragment thereof, by the phosphatase. Synthetic substrates are also known as for example, p-nitrophenyl phosphate, 3-0-methyl-1-fluoroscein monophosphate or fluorosein diphosphate.

Another aspect of the present invention includes a method to identify compounds capable of regulating hematopoietic cell function, the method comprising: (1) contacting a

putative regulatory reagent with PTP1C under conditions suitable for maintaining the enzymatic activity of the PTP1C; and (2) identifying whether the compound is capable of regulating hematopoietic cell function by determining the activity of the PTP1C after the step of contacting. In one embodiment, the method is performed in vitro by: (1) contacting a putative regulatory compound with PTP1C in a container containing suitable buffer and phosphorylated Fyn peptide; and (2) measuring release of [^{32}P] from the Fyn peptide. In another embodiment, the method is performed in a cell-based assay by: (a) contacting a B cell having PTP1C, membrane-bound immunoglobulin and membrane-bound Fc receptor with a putative regulatory compound; (b) contacting the cell with an antibody capable of co-ligating the membrane-immunoglobulin with the membrane-bound Fc receptor; (c) lysing the cell; and (d) determining the state of phosphorylation of an intracellular molecule. Suitable intracellular molecules to measure the phosphorylation of include, but are not limited to, CD19, Syk, Lyn, Fyn, PI-3 kinase, Igα, Igβ, FcεRI β, FcεRI γ, FcγRIIA, Shc and/or PLC γ.

In another embodiment of a cell based assay, the method is performed by: (a) contacting a basophil having PTP1C and membrane-bound Fc receptor with a putative regulatory compound; (b) coligating activating and inhibitory receptors on the cell surface, such as the BCR and FcγR or CD16 and NKIRp59 or Ly49; (c) lysing the cell; and (d) determining the state of phosphorylation of an intracellular molecule. Suitable intracellular molecules to measure the phosphorylation of include, but are not limited to, PI-3 kinase, Fcε RI γ, FcγRIII and/or PLC γ According to the present invention, methods to detect the state of phosphorylation of an intracellular molecule include ELISA assays or FACS analysis. Preferably, a lysed cell can be contacted with an antibody specific for an intracellular molecule bound to an ELISA plate. The plates can be washed and then contacted with an anti-phosphotyrosine antibody bound by a detectable label (e.g., fluorescein, radioisotope or alkaline phosphatase). The amount of anti-phosphotyrosine antibody bound to the ELISA plate can be measured to determine the extent of phosphorylation or dephosphorylation of an intracellular molecule by a putative regulatory compound.

Another aspect of the present invention includes a method to identify a treatment for abnormal hematopoietic cell function, comprising: (1) isolating basophils from a patient; (2) contacting the basophils with ligands including Fab' IgG antibody and whole IgG antibody; and (3) diagnosing abnormal hematopoietic cell function by determining PTP1C activity in the basophils after the step of contacting with the ligand. Preferably, the basophils used with the present method are isolated from human patients.

Another aspect of the present invention includes a method to identify compounds capable of regulating signal transduction using a cell-based assay. In one embodiment, a cell-based assay of the present invention useful for the identification of compounds capable of

modulating (either activating or inhibiting) signal transduction includes the steps of: (a) contacting a cell with a test compound in such a manner that the compound is capable of entering the cell; (b) incubating the compound and the cell so as to allow association of the compound with appropriate intracellular molecules and optionally aggregating cell surface molecules (c) lysing the cell; and (d) determining the state of phosphorylation of an intracellular molecule bound to the compound. Suitable cells include, but are not limited to, mammalian cells. Suitable intracellular molecules include, but are not limited to, src-family tyrosine kinase, in particular Cd19, Lyn, Fyn, Lck, Blk, Syk, Yes, Btk, Hck, Src, and Zap70. Methods to detect the state of phosphorylation of an intracellular molecule include ELISA assays or FACS analysis. Preferably, a lysed cell can be contacted with an antibody specific for an intracellular molecule bound to an ELISA plate. The plates can be washed and then contacted with an anti-phosphotyrosine antibody bound by a tag as described in detail above. The amount of anti-phosphotyrosine antibody bound to the ELISA plate can be measured to determine the extent of phosphorylation and, therefore stimulation, of an intracellular molecule by the putative stimulatory compound. Another method for determining the state of phosphorylation of the intracellular molecule includes contacting an antibody specific for an intracellular molecule with the lysed cells in solution. An anti-phosphotyrosine antibody can be added to the solution and analyzed by FACS analysis. The anti-phosphotyrosine antibody can have a fluorescent tag or a second antibody having a tag can be added to the solution.

In yet another embodiment, a cell-based assay of the present invention can include the steps of: (a) contacting a cell with a putative inhibitory compound; (b) incubating the compound with the cell so as to allow the compound to bind intracellular molecules; (c) ligating membrane-bound receptors on the surface of the cell; and (d) determining the ability of a putative inhibitory molecule to inhibit calcium mobilization in the cell. Suitable cells include, but are not limited to, mammalian cells. Suitable putative regulatory molecules of include mimetopes of Syk-selective, Shc-selective or PI-3K-selective peptides of the present invention. Calcium mobilization can be measured using the methods generally described in Justement et al., *J. Immunol.* 143:881-886, 1989.

In yet another embodiment, a cell-based assay of the present invention can include the steps of: (a) contacting a cell with a putative inhibitory compound; (b) incubating the compound with the cell so as to allow the compound to bind intracellular molecules; (c) ligating membrane-bound receptors on the surface of the cell; and (d) determining the ability of a putative inhibitory molecule to inhibit phosphorylated phosphoinositide formation in the cell. Suitable cells include, but are not limited to, mammalian cells. Formation of phosphorylated phosphoinositides can be measured using the method generally described in Ransom et al. (*J. Immunol.* 137:708-715, 1986).

In a preferred embodiment, the cells used in a cell-based assay of the present invention are permeabilized prior to contacting the cells with a putative inhibitory compound. Techniques to permeabilize cells for use in an assay of the present invention are known in the art.

The present invention also provides methods and kits to identify agents capable of inhibiting or potentiating the various interactions described herein, such as those involving ITIM sequences, of ITIM-activated PTPs. These assays range from simple competitive binding assays, to assays which detect modulation of intracellular signal transduction in a whole cell system.

Exemplary kits kits include: (1) PTP1C protein; (2) a substrate capable of being dephosphorylated by the PTP1C; (3) a means for detecting the dephosphorylation of the substrate; and (4) whereby the ability of a putative regulatory compound to alter the activity of the PTP1C is determined by the detecting means. It is within the skill of one in the art to modify a kit of the present invention to identify regulatory compounds by, for example, adding to the kit appropriate buffers in which to perform a dephosphorylation assay. Suitable substrates for use with a kit of the present invention include, phosphorylated, CD19, Fyn, Lyn, Blk, Syk, Yes, Lck, Btk, Hck, Src, Zap70, Igα, Igβ, FcεRI β, FcεRI γ, FcγRIIA, CD3ε, TCR-zeta, Shc, PI3 kinase, and/or PLC γ. A suitable means for detecting the dephosphorylation, useful with a kit of the present invention, includes the methods of detection generally described in Example 5.

The present invention also includes antibodies capable of selectively binding to PTP1C, PTP1D, ITIM-p160 or ITIM-p70. Binding can be measured using a variety of methods known to those skilled in the art including immunoblot assays, immunoprecipitation assays, enzyme immunoassays (e.g., ELISA), radioimmunoassays, immunofluorescent antibody assays and immunoelectron microscopy; see, for example, Sambrook et al., ibid.

Antibodies of the present invention can be either polyclonal or monoclonal antibodies. Antibodies of the present invention include functional equivalents such as antibody fragments and genetically-engineered antibodies, including single chain antibodies, that are capable of selectively binding to at least one of the epitopes of the protein or mimetope used to obtain the antibodies. Preferred antibodies are raised in response a peptide comprising a portion of ITIM-p160 or ITIM-p70 or a fusion protein produced from a nucleic acid molecule encoding full-length ITIM-p160 or ITIM-p70, or mimetopes thereof.

Still another aspect of the present invention relates to the use of such regulatory compounds as described above in the treatment and/or prophylaxis of various hematopoetic disorders.

The development of an immune response requires the activation of immune cells by extracellular signals. In the case of lymphocyte antigen receptors, antigen recognition results in the activation of intracellular protein tyrosine kinases, particularly members of the Src family kinases, as well as the ZAP-70/syk kinases, resulting in increases in tyrosine phosphorylation. For example, signal transduction via the TCR or BCR results in the phosphorylation of critical tyrosines within the cytoplasmic domains of the TCR CD3/ζ chain complex or the a and b chains of the BCR. The critical tyrosines are located in ITAM sequences. In addition phosphoinositide (PI) hydrolysis is initiated leading to elevation of cytoplasmic free calcium which results in B cell proliferation, differentiation, and antibody secretion (D'Ambrosio et al. (1995) Science 268:293).

The activation of protein tyrosine kinases occurs upon stimulation of many different receptor types. One possible outcome of increases in phosphorylation is the modulation of the activation state of the substrate. Phosphorylation may also lead to changes in the interaction of a protein with upstream or downstream elements in a signal transduction pathway. For example, src homology 2 (SH2) domains found on numerous cytosolic signaling molecules mediate direct interaction with tyrosine-phosphorylated protein tyrosine kinases (PTKs) (Songyang and Cantley (1995) TIBS 20:470). The SH2 domain is a globular domain of approximately 100 amino acids that has a pocket that directly binds the phosphotyrosine moiety of phosphopeptides. SH2 domains have been identified for a wide range of molecules which can be divided into two classes. One class contains proteins which contain functional or enzymatic domains such as src family tyrosine kinases, phospholipase Cγ, GTPase activating protein, two protein tyrosine phosphatases (PTP1C and PTP1D), transcription factor ISGF-3, and the putative GDP-GTP exchanger, Vav. The other class of proteins lack any known catalytic domain and are composed almost exclusively of SH2 and SH3 domains. Members of this group include Crk, GRB2, and Nck. Some of these proteins seem to function as adapters to link transducer molecules to downstream effector molecules (Li et al. (1994) Mol. Cell Biol. 14:509).

Many different cellular receptors also result in the activation of PI3 kinase. PI3 kinase binds to SH2 domains and may lead to PLCγ translocation to the plasma membrane and its activation. PLC activation results in PI turnover and the production of diacylglycerol and inositol 1,4,5-triphosphate (InsP3). IP3 is known to lead to increases in intracellular Ca2+ and result in cell activation, including alterations in gene transcription. For example, production of IP3 can result in release of intracellular calcium, which can then lead to activation of calmodulin kinase II, which can then lead to serine phosphorylation of a DNA binding protein referred to as ets-1 proto-onco-protein. Diacylglycerol is capable of activating the signal transduction protein, protein kinase C which affects the activity of the AP1 DNA

binding protein complex. Signal transduction pathways can lead to transcriptional activation of genes such as c-fos, egr-1, and c-myc.

Downmodulation of immune responses also involves signal transduction. Immune complex-mediated inhibition of antibody production has been shown to depend on coligation of the BCR and the receptor for the Fc region of IgG (FcγRIIB1). Mutational analysis of a gene encoding an FcγRIIB1 has revealed that a 13 amino acid motif in the cytoplasmic domain, is required for negative signaling (Muta et al (1994) Nature 368:70; Amigorena et al (1992), Science 256:1808-1812; and Fridman et al., Immunological Reviews 125:49-76, 1992). This sequence has been termed immune receptor tyrosine-based inhibitor motif (ITIM) (D'Ambrosio et al. (1995) Science 268:293). ITIM motifs have been identified in several receptors, including Ly49, NKIRp58, Fcg receptors, and CD22. An ITIM sequence alignment is shown in Figure 1. In addition SH2 binding ITIMs have been described in the mouse erythropoetin receptor (EPOR), the human IL-2bR, the human IL-3bR, and the c-kit receptor (Thomas (1995) J. Exp Med. 181:1953). In contrast to the ITIM motifs present in NKIRp58, Ly49, Fcg receptors, and CD22 which bind to the COOH terminal SH2 domain of PTP, the ITIM motifs present in the latter group of receptors bind to the NH2 terminal SH2 domain.

In the generation and propagation of intracellular signals, protein-protein interactions are critical in mediating the interactions of upstream and downstream components. For example activation of B cells via the BCR has been found to result in the rapid association of CD22, the transmembrane lectin, with the BCR resulting in the tyrosine phosphorylation of CD22. Phosphorylated CD22 associates with PTP1C via three distinct phosphotyrosine residues (Thomas (1995) J. Exp. Med. 181:1953). PTP1C is thought to play a special role in the downmodulation of the immune response in part based on the phenotype of motheaten mice, which lack PTP1C. Postulated ways by which PTP1C may function include keeping the BCR inactivated before stimulation and/or regulating the threshold with which the BCR responds to antigen (Thomas, supra). Another example of phosphotyrosine mediated recognition is illustrated by the B cell coreceptor, CD19. CD19 is rapidly phosphorylated upon BCR ligation. The phosphorylation of CD19 on tyrosines 484 and/or 515 initiates the recruitment and activation of PI-3 kinase via the SH2 domains of the PI3 kinase p85 subunit (Tuveson et al (1993) Science 260:986). As disclosed for the first time herein, CD19 is also a substrate for PTP1C and the dephosphorylation of CD19 by PTP1C appears to be important in immune cell inhibition.

Without wishing to be bound by theory, it is believed that inhibition of antibody production by immune complex mediated BCR-FcγRIIB1 co-ligation is mediated by a protein tyrosine phosphatase (PTP), for example PTP1C or PTP1D. Upon ligation of the Fcγ

RIIB1, PTP non-covalently associates with the tyrosine phosphorylated ITIM motif of the Fc γRIIB1 by the PTP carboxyl SH2 domain. The binding of the PTP to the ITIM motif activates the enzyme. The activated PTP is co-localized with the BCR-FcγRIIB1 complex by virtue of its association with the ITIM motif of the FcγRIIB1. The PTP then interacts with its substrate and dephosphorylates residues critical for activation. Contemplated substrates include one or more subunits of the receptor (e.g., CD19, Ig-α, Ig-β, Lyn and/or Syk). Alternatively, the PTP could interact with other cellular signaling molecules, such as Shc or phosphotidylinositol-3 (PI-3) kinase. In a preferred embodiment, ITIM-activated PTP1C interacts with and dephosphorylates CD19. Dephosphorylation of one or more of the foregoing molecules can result in the inactivation of one or more signal transduction pathways that stimulate antibody production by the cell. Accordingly, ITIM mimetics can be used to modulate BCR signaling by altering the enzymatic and localization profile of such phosphatases as PTP1C and PTP1D.

The present invention also provides for a method of modulating the activation of natural killer cells. NK cells are important in resistance to malignant cells and in certain viral and microbial infections. NK cells express cell surface receptors for MHC class I proteins (NKIRp58 or Ly49, for example). Engagement of these receptors with their ligands leads to a general inhibition of NK cell cytotoxic programs. Similar receptors can be expressed on T cell subsets, and their engagement results in inhibition of different effector functions initiated by stimulation of the CD3/TCR complex. While the human NKIR genes belong to the Ig gene superfamily, the equivalent mouse NKIR belong to a family of dimeric lectins. Despite these distinct evolutionary origins, both the HLA-C-specific human p58.183 receptors and the H-2Dd- and H-2Dk-specific mouse Ly49A receptors recruit the same protein tyrosine phosphatases, PTP1C and PTP1D, upon phosphorylation of critical intracytoplasmic tyrosine residues. These results document a common pathway by which diverse NKIR can down-regulate the signals leading to NK and T cell activation programs. These findings also further define the sequence recognition requirement of the immunoreceptor tyrosine-based inhibitory motif (ITIM) expressed in both human and mouse NKIR, and which was initially described in FcγRIIB1.

NK cells are cytotoxic lymphocytes which are capable of inducing the lysis of a variety of target cells. One mechanism by which this is accomplished leads to lysis of antibody-coated target cells via the activation of an antibody-dependent cell cytotoxicity program (ADCC). The induction of natural cytotoxicity programs involves lysis of target cells which either do not express, or express in a modified form, MHC class I molecules (Yokoyama, W. M. 1995. Cur. Opinion Immunol. 7:110; Raulet, D. H., and W. Held. 1995. Cell 82: 697; Gumprez, J. E., and P. Parham. 1995. Nature 378: 245). In ADCC, the

engagement of the multimeric CD16 receptor complex activates protein tyrosine kinase (PTK)-dependent pathways leading to the tyrosine phosphorylation of ITAM (immunoreceptor tyrosine-based activation motif) expressed in CD3z and/or FceRIg (Vivier et al. 1991. J. Immunol. 146:206; O'Shea et al. 1991. Proc. Natl. Acad. Sci. USA 88:350; Einspahr et al. 1991. Proc Natl. Acad. Sci. USA. 88:6279; Salcedo et al.. 1993. J. Exp. Med. 177: 1475.). Subsequently, phosphorylated ITAM recruit SH2-tandem PTK such as ZAP-70 and p72Syk (Vivier et al. 1993. Eur. J. Immunol. 23: 1872; Stahls et al. 1994. Eur. J. Immunol. 24: 2491.), which initiate downstream activation events, as described in T cells, B cells and mast cells (Weiss, A. and D. R. Littman. 1994. Cell 76: 263). Recently, the NKIR family of MHC class I -specific receptors capable of inhibiting NK cell activation has been described both in human and in mouse (Karlhofer et al. 1992. Nature 358: 66; Moretta et al. 1993. J. Exp. Med. 178: 597; Phillips et al.1995. Science 268: 40). Human NKIR belong to the Ig superfamily and are characterized, in their extracellular portions by two or three Ig-like domains, corresponding to HLA-C-specific p58 and HLA-B-specific p70/NKB1 receptors respectively (Wagtmann et al. 1995. Immunity 2:439; Colonna, M., and J. Samaridis. 1995. Science 268:405; D'Andrea et al. 1995. J. Immunol. 155:2306). The intracytoplasmic tail is 76-84 amino-acid long, is highly conserved (73% amino-acid identity between human NKIR members), and contains two (I/V)xYxxL stretches originally described as ITAM-like. The integrity of this intracytoplasmic sequence appears to be critical for NKIR-mediated inhibition. Indeed, a naturally-occurring form of the molecule, truncated 2 amino-acid carboxy-terminal of the first Y residue, mediates NK cell activation rather than inhibition. Yet, the unusual length of the spacer region between both (I/V)xYxxL, spanning 26 amino-acids instead of the classical 6-8 amino-acids present in CD3/TCR-, FcR- and BCR-associated molecules, may suggest that an independent, and possibly different, function for these two stretches. Along this line, a single intracytoplasmic (I/V)xYxxL sequence is included in FcγRIIB1 ITIM, which has been shown to mediate inhibition of B cell activation induced by surface Ig (D'Ambrosio et al. 1995. Science 268:293). Studies in transfected cells further indicated that the FcgRIIB1 ITIM can inhibit cell triggering mediated via the CD3/TCR as well as the FceRI complexes (Daèron et al. 1995. Immunity 3:1). The molecular dissection of FcgRIIB1-induced inhibition revealed that the Y309 residue of mouse FcgRIIB1 ITIM is phosphorylated upon BCR-FcγRIIB1 co-engagement, and recruits several proteins, including PTP1C (D'Ambrosio et al. 1995. Science 268:293). The PTPase PTP1C (HCP, SHP, SH-PTP1) is a hematopoietic specific-PTPase expressing two tandem SH2 domains linked to a C-terminal catalytic domain (Stone, R. L., and J. E. Dixon. 1994. J. Biol. Chem. 269:31323). PTP1C is involved in many inhibitory signaling pathways, such as the termination of erythropoietin receptor as well as Kit/SCF receptor signals. In addition, the B cell surface molecule CD22 contains 3 I/VxYxxL stretches which can bind PTP1C upon

tyrosine phosphorylation, and regulates B cell activation programs (Doody et al. 1995. Science 269:242; Campbell, M. A., and N. R. Klinman. 1995. Eur. J. Immunol. 25:1573). Remarkably, (I/V)xYxx)(L/V) is expressed in the mouse lectin NKIR Ly-49A, C, F. G1 and G4, as well as in the human lectin receptors NKG2A and B (See Figure 1). These observations are compatible with the presence of putative ITIM-like sequences in human and mouse NKIR, which despite their distinct genetic origin (Ig superfamily vs. lectin), could exert their inhibitory function through the recruitment of PTPases, such as PTP1C.

In accordance with the foregoing mechanisms, one embodiment of the present invention includes a cellular system where an activating receptor is coligated with a negative regulatory receptor, for example an FcγRII is co-ligated with a B cell receptor which is regulated by the FcγRII. The cell is then contacted with a regulatory reagent capable of binding to a protein including PTP1C, PTP1D, ITIM-p160 and ITIM-p70. Preferably, the Fc γRII contains the amino acid sequence TAENTITYSLLKH, GAENTITYSLLMH, preferably in which the tyrosine residue is phosphorylated. More preferably the FcγRII contains the amino acid sequence ITYSLL, and even more preferably IXYXXL, preferably the tyrosine residue is phosphorylated. In particulary preferred embodiments they reagent comprises the core sequence ((I/V)XYXX(L/V), in more preferred embodiments the tyrosine residue is phosphorylated.

Also in accordance with the foregoing mechanism, another embodiment of the present invention includes a method to regulate antibody production, comprising contacting a cell, having a receptor selected from the group consisting of a B cell receptor, an FcγRIII, an Fcε RI and/or an FcγRII, with an effective amount of a regulatory reagent that is capable of binding to a protein including PTP1C, PTP1D, ITIM-p160 and ITIM-p70, in which the protein is capable of altering the biological response resulting from the co-ligation of a combination of receptors selected from the group consisting of a B cell receptor and an Fcγ RII, an FcεRI and an FcγRII, and an FcγRIII and an FcγRII, thereby resulting in the modulation of antibody production, and/or proliferation. If antibody production is inhibited, the present method is particularly useful for protecting an animal from diseases such as allergic responses and autoimmune diseases. If antibody production is stimulated, the present method is particularly useful for protecting an animal from diseases such as AIDs and diseases against which an animal can be immunized (i.e., diseases caused by an infectious agent).

A separate aspect of the present invention relates to the recognition that one can regulate the activity of a signal transduction molecule in a cell by contacting a cell with a regulatory reagent of the present invention. In one embodiment, a method for regulating hematopoietic cell function comprises contacting a cell with an effective amount of a

regulatory reagent of the present invention that is capable of binding to a protein selected from the group consisting of PTP1C, PTP1D, ITIM-p160 and ITIM-p70, in such a manner that the activity of the protein is altered (e.g., inhibited or stimulated) resulting in regulation of hematopoietic cell function. In another embodiment, a method for regulating hematopoietic cell function comprises contacting a cell with an effective amount of a regulatory reagent of the present invention that is capable of altering the concentration of a molecule selected from the group consisting of PTP1C, PTP1D, ITIM-p160 and ITIM-p70 in the cell, thereby resulting in the regulation of hematopoietic cell function

It is yet another aspect of the present invention to utilize the subject ITIM peptide or peptidomimetics or other ITIM mimetic to modulate intracellular signaling pathways. As used herein, an "ITIM mimetic" refers to an agent capable of agonizing or antagonizing a signal transduction activity mediated by an ITIM sequence of a receptor. The ITIM peptides and peptidomimetics described above are of course ITIM mimetic, as are agents discovered in the drug screening assays described above. Moreover, other SH2 binding agents described in the art, while perhaps not as optimal as the subject ITIM peptides for, e.g., activating PTP1C or PTP1D, may nevertheless be used to modulate ITIM-dependent activities. For instance, it is contemplated that the ITIM mimetic can be similar to the phosphotyrosine benzodiazepine peptidomimetics described in PCT application WO 95/25118 to Bachovchin, and the phosphotyrosine peptides described the PCT application WO 94/08600 to Shoelson. both of these publications describe tetra peptides corresponding to pYXXL, e.g. not including amino acid residues in the -2 position as our data suggest. Nevertheless, such other phosphotyrosinecontaining peptides may be capable of interacting with PTP1C, PTP1D, ITIMp160, ITIM p70, and/or other cellular targets with which a native ITIM interacts. These ITIM mimetics may mimic the PTP activation of a native ITIM, or may be agonistic (e.g. inhibitory) of that activation by competitively binding to the PTP1C SH2 domain yet failing to allosterically activate the phosphatase.

In general, an ITIM mimetic can be contacted with a cell, either in vivo (e.g. adminstered to a patient or animal) or in vitro (e.g. employed in cell culture), to affect the responsiveness of a hematopoetic cell to a growth factor, cytokine or other receptor ligand; to modulate proliferation, influence differentiation of cells, or modulate effector functions of cells, such as antibody production, antibody dependent cellular cytotoxicity, cytokine secretion, secretion of other soluble mediators (including preformed mediators), or cytolysis among others. In one aspect of the invention, the subject compound modulates the signal transducing ability of an protein having an enzymatic activity. Exemplary enzymes include cytoplasmic tyrosine kinases (eg. Src, Lck), phosphotyrosine phosphatases, phospholipase Cγ (PLCγ) and the like. Thus, inhibiting or, alternatively, mimicking SH2-mediated interactions

with the compounds of the present invention can modulate the activation of the enzyme, as well as alter the compartmentalization or localization of the enzyme.

The subject compounds may also be utilized as part of therapeutic regimens for the treatment of a variety of IgE mediated diseases, such as inflammatory and allergic reactions implicating degranulation of basophils and mast cells. For example, the compounds of the present invention can be used to control chronic allergic inflammation, as well as in the treatment of asthma.

Briefly, the immediate-type hypersensitivities, such as anaphylaxis, allergic rhinnitis, atopic dermatitis, asthma, allergic bronchopulmonary aspergillosis, hepersensitivity pneumonitis, extrinsic asthma, hay fever, and allergic responses to certain foods or drugs, are mediated primarily by one of the immunoglobulin isotypes, i.e., IgE. In an IgE-mediated allergic response, the allergen binds to the IgE which is bound to receptors (e.g., MIRRs) on the surface of mast cells and basophilic leukocytes (basophils). The binding of the allergen causes crosslinking of the surface IgE molecules and hence the underlying receptors for the Fc portion of IgE (FcεR), thereby triggering cellular degranulation with the release of pharmacologic mediators such as histamine, neutral proteases (e.g. tryptases, chymotryptase, carboxypeptidase), LTC4, LTB4, PGD2, TXA2, PAF, cytokines (e.g. IL-4 or TNFα), the slow-reacting substance of anaphylaxis (SRA), and serotonin. The release of these mast cell and basophil products causes the pathological reactions and symptoms of allergy.

Another embodiment of the present invention is includes a method to regulate the release and/or production of mast cell or basophil mediators such as, histamine, IL-5 and/or IL-8, or other mediators of hypersensitivity responses comprising contacting a basophil or mast cell with an effective amount of a regulatory reagent that is capable of binding to a protein including PTP1C, PTP1D, ITIM-p160 and ITIM-p70, in which the protein is capable of altering the biological response resulting from the co-ligation of an FcγRIII and/or an Fcε RI with an FcγRII, thereby resulting in the inhibition of histamine, IL-5 and/or IL-8 production or release. The step of regulating includes stimulating or inhibiting IL-5 or IL-8 production, and inhibiting histamine production. If histamine or cytokine production is inhibited, the present method is particularly useful for protecting an animal from allergic or other immune responses. If IL-5 is stimulated, the present method is particularly useful for treatment of diseases in which antibody production is needed, such as in protection of an animal from immunodeficiency diseases or infectious diseases.

Yet another embodiment of the present invention features the use of an anatagnostic ITIM mimetic, or other agent which inhibits the inactivating phosphatase PTP1C or PTP1D, as an adjuvant. By interferring with the ability of, for example, PTP1C to downregulate antigen-induced signaling in lymphocytes, such agents can be used to enhance the humoral

and/or cellular response to an antigen co-administered with such a compound. For example, ITIM peptides which bind to PTP1C but which fail to activate the phosphatase or inhibit its localization may be useful adjuvants. Likewise, compounds which inhibit the phosphatase activity directly, such as which may be discovered in the assays set out above, may also serve as adjuvants.

By similar principals, these adjuvants may also be use to enhance immunoresponsiveness in the treatment of a variety of immunodeficiencies.

The invention also relates to agents which can be utilized as part of therapeutic regimens for the treatment of a variety of IgE mediated diseases, such as inflammatory and allergic reactions implicating degranulation of basophils and mast cells. In one embodiment the present invention provides for ITIM peptides or nucleic acids, in particular ITIM pharmaceutical preparations which are capable of modulating the activation of basophils and/or mast cells by modulating FcεR signaling. In another embodiment, the subject invention provides bispecific antibodies for cross-linking Fcε receptors (FcεR) with FcγR in order to inhibit signaling from FcεR engaged by IgE. For example, the bispecific antibodies of the present invention can be used to control chronic allergic inflammation, as well as in the treatment of asthma.

Briefly, the immediate-type hypersensitivities, such as atopic dermatitis, extrinsic asthma, hay fever, and allergic responses to certain foods or drugs, are mediated primarily by one of the immunoglobulin isotypes, i.e., IgE. In an IgE-mediated allergic response, the allergen binds to the IgE which is bound to receptors (e.g., MIRRs) on the surface of mast cells and basophilic leukocytes (basophils). The binding of the allergen causes crosslinking of the surface IgE molecules and hence the underlying receptors for the Fc portion of IgE (Fc εR), thereby triggering cellular degranulation with the release of pharmacologic mediators such as histamine, the slow-reacting substance of anaphylaxis (SRA), and serotonin. The release of these mast cell and basophil products causes the pathological reactions and symptoms of allergy.

Antigen-mediated aggregation of the high-affinity receptor for immunoglobulin E, Fc εRI, results in the activation of multiple signaling pathways, leading to the release of mediators of the allergic response. However, as described in the appended examples, coligation of the FcεRI with FcγR results in down regulation of FcεR signal transduction. Not wishing to bound by any particular theory, recruitment of PTPs, such as PTP1C by the coligated FcεR may cause dephosphorylation of the ITAM motif tyrosines in the β and γ subunits of FcεRI and thereby antagonize association of the tyrosine kinase Syk with the phosphorylated receptor or may block FcεR signaling by inhibiting PI3Kinase activation by a CD19 analog. The former association is mediated by the SH2 domains of Syk and is believed

to be critical for activating signaling pathways resulting in mediator release. Accordingly, disruption of the FcεR-mediated signal pathway by a compound of the present invention can provide for prophylaxis and/or direct treatment of diseases or injuries implicating degranulation of such cells.

Moreover, it is becoming increasingly apparent that both the basophil response and mast cell response are up-regulated by a number of cytokines. Consequently, in addition to inhibiting the Fcε receptor signal, the subject compounds can further be used in the treatment of, for example, allergic lesions by selective modulation of signal transduction pathways induced by cytokines.

One function characteristic of every antibody molecule is specific binding to an antigenic determinant. Antibodies *in vivo* are bivalent and monospecific, containing two identical antigen binding sites. The specific binding of antigen by an antibody molecule is determined by the antibody's structure of the variable regions (Fab) of both heavy and light chains. Antibodies having dual specificities have been prepared by subjecting antibodies of different specificities to a selective cleavage of the disulfide bridges that link the two heavy chains together. Antibody half-molecules are then reassociated under neutral pH to produce the hybrid antibodies having dual specificities. Such approaches can be employed to make a bispecific antibody which is capable of crosslinking the FcεR and FcγR.

Nisonhoff et al., (1962) *Nature* 194:355, describe one applicable method for the *in vitro* production of a bispecific antibody molecule. According to the Nisonoff method, the monospecific anti-FcεR and anti-FcγR antibodies are treated with pepsin to remove the Fc portion of each antibody, leaving two antigen-binding sites (Fab) covalently linked by a single disulfide bond. This bond is then split under reducing conditions and two antibody fragment pools combined and reassociated together under oxidizing conditions to produce a bispecific antibody.

In Brennan et al. (1985) *Science* 299:31, a chemical procedure is described for preparing bispecific antibody fragments from monoclonal antibodies. In this procedure, a modification of the Nisonoff technique is used in cleaving the Fab fragments, followed by reconstituting the half-fragments to form the bispecific antibody molecule. The Fab fragments are reduced in the presence of sodium arsenite to stabilize vicinal dithiols and impede intramolecular disulfide formation. The other modification involved activating the thiols of one of the half-Fab fragments as a thionitrobenzoate derivative. By this process, a bispecific antibody can be produced from anti-FcεR and anti-FcγR Fabs.

Liu et al. (1985) *PNAS* 82:8648, discloses a chemical procedure for forming a bispecific antibody. By this method, anti-FcεR and anti-FcγR antibodies can be covalently

linked. The anti-FcεR and anti-FcγR antibodies are first reacted with N-suc-cinimidyl-3-(2-pyridyldithio) propionate (SPDP). Thiol groups are attached to the cleaved anti-FcεR antibody using 2-iminothiolane. Then the two modified half-antibodies, anti-FcεR and anti-FcγR, are mixed to covalently link the two antibodies.

In still another embodiment, the {anti-FcεR x anti-FcγR} bispecific antibodies of the present invention can be produced from hybridomas. The preparation of bispecific monoclonal antibodies by fusion of antibody-producing hybridoma cells is described in Milstein and Cuello, (1983) *Nature* 305:537 and PCT application, WO83/03679. By this method, two hybridomas (expressing anti-FcεR and anti-FcγR antibodies respectively) are fused to produce hybrid hybridomas. These hybrid hybridomas secrete can be selected for those which produce predefined bispecific monoclonal antibodies. The bispecific monoclonal antibodies produced by hybrid hybridomas are complete molecules, containing the Fc region as well as the antigen-combining sites.

It is noted that pharmaceutical preparations of the bispecific monoclonal antibodies described herein could be formulated into pharmaceutical preparations using any of the methods described above.

The following examples are provided for the purposes of illustration and are not intended to limit the scope of the present invention.

Examples

Example 1. This example describes the identification of PTP1C, ITIM-p160 and ITIM-p70 as ITIM binding molecules in co-precipitation studies.

Non-phosphorylated (ITIM peptide) and tyrosine phosphorylated murine ITIM peptides (pITIM) having the sequences TAENTITYSLLKH and TAENTIT(p)YSLLKH, respectively, were synthesized by Macromolecular Resources, Inc. (Ft. Collins, CO).

A20 B lymphoma cells were loaded with [^{35}S]-Methionine using the method generally described in Cold Spring Harbor Labs Press, 1989 Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Press, 1988. The [^{35}S]-Methionine A20 cells were used to prepare several different samples. The first sample included 1 x 10^8 per ml unstimulated A20 cells. The second sample included 1 x 10^8 per ml A20 cells stimulated with intact (80 μg/ml) rabbit anti-mIg antibodies (Zymed, San Franscisco, CA) for 2 min at 37°C. The third sample included 1 x 10^8 per ml A20 cells stimulated with F(ab')$_2$ (50 μ g/ml) of the rabbit anti-mIg antibodies. The cells in the samples were immediately lysed in

1% NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 2.0 mM Sodium Orthovanadate, 10 mM Sodium pyrophosphate, 0.4 mM EDTA, 10 mM NaF, 1 mM Phenylmethylsulfonyl fluoride, and 2 μg/ml each of aprotinin, leupeptin, and a-1-antitrypsin). Peptide adsorbates were produced by incubating separate 0.4 ml of postnuclear 1% NP-40 lysates of unstimulated, intact or $F(ab')_2$ rabbit anti-mIg antibody-stimulated [^{35}S]-Methionine labeled A20 cells (20 x 10^6 cells per sample) with either ITIM and pITIM peptide (20 μg) coupled Sepharose beads (10 μl of 50% slurry) (Pharmacia, Piscataway, NJ) for 1 hr at 4°C. The absorbed material was eluted with 2.2% formic acid (0.1 ml) which was subsequently eliminated by lyophilization. The peptide adsorbates were boiled for 5 min in SDS reducing sample buffer and fractionated by 10% SDS-PAGE. Any [^{35}S]-Methionine eluted from the peptide-bound beads were identified by autoradiography.

No proteins were identified in the samples absorbed to the ITIM peptide. Three proteins, however, were identified in the unstimulated and stimulated cell lysate samples incubated with the pITIM peptide. The relative molecular weights of the proteins were 160, 70 and 65 kDa. The protein having a molecular weight of 160 is referred to herein as ITIM-p160. The protein having a molecular weight of 70 is referred to herein as ITIM-p70.

Example 2. This example describes the amino acid sequencing of the tyrosine phosphorylated pITIM-binding 65 kDa protein.

Samples of the three pITIM-binding proteins described in Example 1 were isolated from NP-40 lysates of 5 x 10^9 A20 cells by incubating the lysate with 100 μg pITIM peptide and eluting any peptide-bound protein using 50 mM (100 μl) p-NPP (a phosphotyrosine analog). The eluted proteins were resolved on a 10% SDS-PAGE gel and the 65 kDa band was transferred onto Immobilon-P membrane (Millipore Corp., Bedford, MA). The proteins isolated on the membrane were subjected to N-terminal sequence analysis using a PROCISE Applied Biosystems amino acid sequencer (Applied Biosystems, CA). The N-terminal 17 amino acid sequence was found to be a perfect match for the phosphotyrosine phosphatase PTP1C (also known as HCP or SH-PTP1).

Example 3. This example describes immunoblot experiments using antibodies specific for PTP1C to confirm the amino acid sequencing data.

pITIM peptide adsorbates were prepared as described in Example 1. The adsorbates were resolved by SDS-PAGE and the proteins were electrophoretically transferred to Immobilon-P membranes. Anti-PTP1C antibodies were prepared by immunizing rabbits with the N-terminal portion of PTP1C protein cleaved from a bacterially expressed GST fusion

protein (residues 1 to 193). The polyclonal antiserum was used to probe the membrane containing the transferred protein using techniques standard in the art. The resulting immunoblots were developed with an enhanced chemiluminescence (ECL) detection system (Amersham, Buckinghamshire, England).

A protein having the molecular weight of 65 kDa was identified using the anti-PTP1C antiserum. In addition, equal amounts of PTP1C were present in pITIM peptide adsorbates of whole and F(ab')$_2$ rabbit anti-mIg stimulated and unstimulated A20 cells, thus indicating that PTP1C association the pITIM peptide does not require receptor mediated modification of the PTP1C enzyme.

Example 4. This example describes the identification of PTP1C binding to FcγRIIB1 *in vivo*.

FcγRIIB1 was immunoprecipitated from 0.1 ml of postnuclear 1% NP-40 lysates of unstimulated, intact or F(ab')$_2$ rabbit anti-mIg antibody stimulated A20 cells (50 x 10^6/sample) by incubating the lysates with 2.4G2 (30 μg) coated Sepharose beads (25 μl of 50% slurry) for 15 min at 4°C. The immunoprecipitated proteins were resolved by SDS-PAGE and transferred to blotting paper using the methods described in Example 2. The blotting paper was sequentially immunoblotted with 1:1000 dilution of ascites containing anti-FcγRIIB antibody, 1:1000 dilution of anti-phosphotyrosine antibody (Ab2, Oncogene Science, Uniondale, NY), and 1:1000 dilution of anti-PTP1C antiserum.

The immunoblotting results obtained using the anti-phosphotyrosine antibody indicated that FcγRIIB1 is tyrosine phosphorylation upon stimulation of the A20 with intact anti-mIg antibodies. No tyrosine phosphorylation stimulation was detected in the unstimulated cells or in those cells incubated with F(ab')$_2$ anti-mIg antibody. Immunoblotting results obtained using the anti-FcγRIIB antibody indicated that stimulation of the A20 cells with intact anti-mIg antibody had no effect on the levels of FcγRIIB1 in the immunoprecipitations.

The immunoblotting results obtained using the anti-PTP1C antibody indicated that PTP1C co-immunoprecipitated with FcγRIIB1 only in cells stimulated with intact anti-mIg antibody. The stimulation with F(ab')$_2$ anti-mIg antibody did not induce PTP1C association while inducing comparable global protein tyrosine phosphorylation.

Taken together, the results of these immunoprecipitation studies indicate that BCR-Fc γRIIB1 co-ligation is necessary for FcγRIIB1 tyrosine phosphorylation because only whole anti-mIg antibody induced FcγRIIB1 tyrosine phosphorylation. In addition, the results indicate that tyrosine phosphorylation of FcγRIIB1 leads to recruitment of PTP1C to the activated co-ligated FcγRIIB/BCR complex.

Example 5. This example describes the identification of the situs on PTP1C which interact with pITIM peptide.

A. Fusion Protein Analysis

Bacterially expressed GST fusion proteins of PTP1C amino or carboxyl SH2 domain (Clark et al., pp. 123-126, 1992, *Science*, Vol 258) were incubated with lysates of unstimulated, whole and $F(ab')_2$ anti-mIg stimulated A20 cells prepared according to the method described in Example 1. Proteins contained in the PTP1C fusion protein adsorbates were resolved by SDS-PAGE and transferred to blotting paper as described in Example 2. The blotting paper was immunoblotted with the anti-phosphotyrosine and anti-FcγRIIB1 antibodies as described in Example 3.

The immunoblotting results obtained using both the anti-FcγRIIB1 and anti-PTP1C antibodies indicated that the fusion protein containing the carboxyl but not the amino SH2 domain of PTP1C was able to precipitate the tyrosine phosphorylated FcγRIIB1 from lysates of whole anti-mIg antibody stimulated cells. The experiments also showed that neither the carboxyl nor the amino SH2 domain of PTP1C bound to FcγRIIB1 from unstimulated or $F(ab')_2$ stimulated cells, thus indicating that the interaction between PTP1C and FcγRIIB1 requires BCR co-ligation and/or co-ligation dependent tyrosine phosphorylation of the Fcγ RIIB1.

B. Peptide Analysis

Upon determination that the carboxyl SH2 domain of PTP1C associates with the tyrosine phosphorylated FcγRIIB1, the association was confirmed using pITIM. Peptide adsorbates were prepared as described in Example 1 by incubating 20 μg PTP1C amino- or carboxyl SH2 domain fusion protein were precipitated with 5 μg pITIM peptide coupled Sepharose beads. Proteins contained in the peptide adsorbates were resolved by SDS-PAGE and the gel was stained with Comassie Blue.

The results from the Comassie Blue stained gel indicated that the PTP1C fusion protein containing the carboxyl SH2 domain bound to the pITIM peptide. The PTP1C fusion protein containing the amino SH2 domain, however, did not bind to pITIM peptide. Thus, the results indicate that PTP1C associates directly with the tyrosine phosphorylated FcγRIIB1 via a carboxyl SH2-pITIM interaction.

Example 6. This example describes the effect of pITIM binding to PTP1C on the PTP1C phosphatase activity by measuring by measuring the release of [^{32}P] from the phosphorylated Lyn autophosphorylation site peptide.

PTP1C was isolated by incubating 1% NP-40 lysates (lacking Sodium Orthovanadate and Sodium pyrophosphate) of unstimulated A20 cells (2 x 10^8) with 250 µg pITIM coated Sepharose beads (100 µl of 50% slurry) using the methods described in Example 1. After incubation for 1 hr at 40°C, the peptide-coated beads were washed twice in 1% NP-40 lysis buffer and once in phosphatase buffer (10 mM Imidazole (pH 7.0), 50 mM EDTA, 0.1% 2-mercaptoethanol, 1 mg/ml bovine serum albumin). The pITIM bound PTP1C was eluted with 300 µl of 50 µM p-NPP in phosphatase buffer. The p-NPP was eliminated from the eluent by dialysis by centrifugation in a Microcon 10 tube (Amicon, Beverly, MA) in 30x volume of phosphatase buffer for 30 minutes at 4°C.

A peptide having the sequence RRLIEDNEYTARQGA (Fyn peptide) was obtained from Macromolecular Resources, Inc. which contains an autophosphorylation site. The Fyn peptide was phosphorylated at the tyrosine residue using [^{32}P]-ATP and the B subunit if the Insulin Receptor Kinase using the method described in Hippen et al., *Biochemistry* 32:12405-12410, 1993. The phosphorylated Fyn peptide was then used to test the phosphatase activity of the PTP1C isolated above. Using the method generally described in Lechleider et al. (*J. Biol. Chem.* 268:21478-21481, 1993), dephosphorylation of the Fyn peptide was performed at 30°C for 15 minutes in the presence of 20 µl of the eluted PTP1C and 30,000 cpm of [^{32}P]-ATP-labelled Fyn peptide in a final volume of 40 µl. The assay was performed in the absence or presence of various amounts on ITIM peptide, pITIM peptide, tyrosine phosphorylated Ig-α (ENL(p)YEGLNLDDCSM(p)YEDI) and FcεRI-β ITAM peptides (DRL(p)YEELNHV(p)YSPI(p)YSEL). The release of [^{32}P] from the substrate was determined as described in Lechleider et al. (*ibid.*).

Three independent experiments were performed as described above, with each sample being performed in duplicate. The results of the phosphatase assays indicated that the addition of pITIM peptide caused a 4-fold increase in the specific activity of the PTP1C enzyme. The ITIM peptide did not affect PTP1C activity, while the tyrosine phosphorylated Ig-α and FcεRI-β ITAM peptides significantly inhibited the release of [^{32}P]. Taken together. the results indicate that the association of PTP1C with pITIM peptide regulates the phosphatase activity of PTP1C by increasing such activity.

Example 7. This example describes the correlation of FcγRIIB1 inhibitory function with tyrosine phosphorylation of the FcγRIIB1 ITIM and association with PTP1C *in vivo* using a mutated FcγRIIB1 which lacks the ITIM tyrosine.

Expression vectors encoding FcγRIIB1 truncated at amino acid residue 314 and containing the wild type ITIM (CT314 WT) and FcγRIIB1 truncated at amino acid residue 314 in which the tyrosine 309 of the ITIM was substituted with alanine (CT314 Y309-A) were transformed into IIA1.6 cells (generally described in Jones et al., *J. Immunol.* 136:348-352, 1986) and are referred to herein as CT314 WT-IIA1.6 cells and CT314 Y309-A-IIA1.6 cells, respectively.

Expression of the CT314 WT and CT314 Y309-A encoded receptor on the surface of their respective cell lines was confirmed as follows. About 1 x 10^6 CT314 WT-IIA1.6 cells or CT314 Y309-A-IIA1.6 cells were stained with biotinylated 2.4G2 antibody and fluoresceinated avidin (Tago Inc., Burlingame, CA) or with fluoresceinated avidin only and analyzed by flow cytometry (FACScan, Becton Dickinson, Richmond, CA) as described in Justement et al., *J. Immunol.* 143:881-886, 1989). The results indicated that CT314 WT-IIA1.6 cells expressed CT314 WT encoded increased receptor numbers over background and CT314 Y309-A -IIA1.6 cells expressed CT314 Y309-A encoded increased receptor number over background.

A. Calcium Assays

The ability of the CT314 WT and CT314 Y309-A encoded receptors to inhibit Ca^{2+} mobilization following co-ligation of the receptors with the BCR were assessed. 1 x 10^6 CT314 WT-IIA1.6 cells and CT314 Y309-A -IIA1.6 were loaded with 5 μM Indo-1 AM (Molecular Probes, Eugene, WA) using methods described in Justement et al., *ibid.* The Indo-loaded cells were then stimulated with either 12 μg/ml of $F(ab')_2$ anti-mIg antibodies or 20 μg/ml intact anti-mIg antibodies and the Ca^{2+} responses were recorded by a flow cytometer (Model 50H, Ortho Diagnostic Systems, Westwood, MA). The % cells responding was evaluated using an appended data acquisition system and the MultiTIME software (Phoenix Flow Systems, San Diego, CA).

The results indicated that stimulation of CT314 WT-IIA1.6 cells with $F(ab')_2$ anti-mIg antibodies induced a rise of $[Ca^{2+}]_i$ which was significantly inhibited when stimulation was performed with whole anti-mIg antibodies, thus indicating that the truncation does not significantly impair FcγRIIB1 negative signaling. In contrast, untransfected or CT314 Y309-A-IIA1.6 cells showed similar late phase Ca^{2+} mobilization following stimulation with whole or $F(ab')_2$ anti-mIg antibodies, thus indicating that the tyrosine of the ITIM is essential for inhibition of Ca^{2+} mobilization. A modest inhibition of Ca^{2+} mobilization was detectable in response to BCR co-ligation with the CT314-Y309-A mutant FcγRIIB1

indicating that other regions within the cytoplasmic domain may play a role in FcγRIIB1 mediated negative signaling.

B. Tyrosine Phosphorylation Studies

FcγRIIB1 were immunoprecipitated from unstimulated, intact (80 μg/ml) or $F(ab')_2$ (50 μg/ml) stimulated IIA1.6 transfected cells (50 x 10^6/samples) using methods described in Example 2. Immunoprecipitated material was collected by formic acid elution as described in Example 2 and after boiling for 5 min in SDS reducing sample buffer, fractionated by 10% SDS-PAGE, electrophoretically transferred to Immobilon-P and subjected to sequential immunoblotting with anti-phosphotyrosine antibody and anti-FcγRIIB1 antibody. Immunoblots were developed with ECL detection system (Amersham).

The results indicated that CT314 WT isolated from cells stimulated with whole anti-mIg antibodies were tyrosine phosphorylated and bound PTP1C protein. Conversely, the CT314 Y309-A receptor was not phosphorylated and did not bind PTP1C following BCR co-ligation.

Taken together with the foregoing calcium assay results, these data indicate that tyrosine phosphorylation of the ITIM is important for PTP1C association as well as FcγRIIB1 mediated negative signaling, suggesting that PTP1C association is essential for FcγRIIB1 function.

Example 8. This example describes FcεRI mediated signaling and PTP1C expression in rat basophilic leukemia cells.

Rat Basophilic Leukemia (RBL) cells that express endogenous Fcε receptor (FcεRI) were transformed with an expression vector encoding FcγRIIB. Expression of PTP1C in the transformed RBL cells was determined by immunoblotting of whole cell lysates using anti-PTP1C antiserum. The immunoblot results indicated that the transformed RBL cells expressed comparable amounts of PTP1C as A20 B cells.

Using the transformed RBL cells described above, the extent of tyrosine phosphorylation following FcεRI aggregation was compared with K46μ B lymphoma cells. K46μ B lymphoma cells (4 x 10^7/ml) were stimulated with 50 μg/ml monoclonal anti-IgM antibody (b-7-6) at 37°C for 3 minutes. RBL cells (1 x 10^6/ml) were incubated overnight with DNP-specific IgE antibody and DNP-BSA antigen (20 ng/ml) for 3 minutes at 37°C. Clarified 1% NP-40 lysates of stimulated or nonstimulated cells were prepared (1 x 10^6 cell equivalents for K46μ; 5 x 10^5 cell equivalents for RBL) and fractionated by SDS-PAGE under reducing conditions. Proteins were transferred to Immobilon, probed by immunoblot analysis using anti-phosphotyrosine specific antibodies and visualized using the ECL method.

The immunoblot results indicate that the transformed RBL cells exhibit protein tyrosine phosphorylation and responses upon FcεRI aggregation using rat IgE anti-DNP antibodies and DNP-BSA antigen.

Example 9. This example shows that human and mouse natural killer cell inhibitory receptors recruit the PTP1C and PTP1D protein tyrosine phosphatases.

Materials And Methods

Peptides. In the mouse, Ly49A has been shown to directly interact with H-2D^d and H-2D^k (Karlhofer, F. M., R. K. Ribaudo and W. M. Yokoyama. 1992. *Nature* 358: 66), whereas in humans, p58.183 interacts with HLA-Cw3 (Moretta, A., M. Vitale, C. Bottino, A. M. Orengo, L. Morelli, R. Augugliaro, M. Barbaresi, E. Ciccone, and L. Moretta. 1993. *J. Exp. Med.* 178: 597). The following peptides were obtained as non-phosphorylated or as tyrosine-phosphorylated. Numbering of amino-acids (subscript number) is from the starting M residue of the coding sequence.

p58.183.1: $D_{294}EQDPQEVT\mathbf{Y}_{303}AQLNH_{308}$

p58.183.2: $R_{318}PSQRTKTPPTDIIV\mathbf{Y}_{333}AELPNA_{339}$

Ly-49A: $S_2EQEVT\mathbf{Y}_8SMVRF_{13}$

CD3ζ1: $YQ_{64}QGQNQL\mathbf{Y}_{71}NELNLGRREE\mathbf{Y}_{82}DVLDKRRGR_{91}$

FcεRIγ: $G_{64}V\mathbf{Y}_{66}TGLSTRNQET\mathbf{Y}_{78}ETLKHEKPPQ_{87}$

The phosphorylated CD3ζ1, FcεRIg, p58.183.1 and p58.183.2 peptides were obtained from Neosystem (France) and synthetized with a N-terminal biotin. Unphosphorylated peptides were synthetized by P. Fourquet at the Centre d'Immunologie de Marseille-Luminy, and were obtained as N-terminal biotinylated (NHS-LC-biotin, Pierce) or not. Biotinylated peptides were coupled to streptavidin-agarose beads (Sigma), and Ly49 peptides were coupled to CnBr-sepharose beads (Pharmacia) as recommended by the manufacturers.

Surface plasmon resonance. SPR measurements were performed on a BIAcore apparatus (Pharmacia). The GST-PTP1C-SH2 (N+C) and GST-ZAP-70-SH2 (N+C) fusion proteins have been previously described (Moretta et al. 1993. *J. Exp. Med.* 178: 597d, and were purified from DH5a lysates as indicated (D'Ambrosio et al. 1995. *Science* 268:293). Recombinant His-tagged full length PTP1D protein was purified on Ni-affinity column. Biotinylated peptides (2 ng) were immobilized on streptavidin microchips, and their immobilization was confirmed by the binding of an anti-phosphotyrosine mAb (4G10, UBI).

Cells and cell lysates. CD3/TCR$^+$ T cells expressing p58.183 NKIR were isolated from peripheral blood lymphocytes and cultured in the presence of recombinant IL-2 (100U/ml) as previously described (12). The human NK cell lines NK3.3 (Kornbluth et al. 1982. *J. Immunol.* 129:2831) and NKL (Kaufman et al. 1995. *Proc. Natl. Acad. Sci. USA* 92:6484) were maintained in RPMI-1640 supplemented with 10% human AB serum and penicillin-streptomycin. Cells were lysed in NP-40 lysis buffer (1% NP-40, 10 mM Tris-HCl, 150 mM NaCl, 1mM EDTA, 1 mM PMSF, 10 mM iodoacetamide, 10 mM NaF, 10 mM Na pyrophosphate, 0.4 mM Na vanadate) for 15 minutes on ice. After removing insoluble material by centrifugation at 12,000 rpm for 15 minutes, samples were either used directly or subjected to affinity purification using peptides bound to beads. Biotinylated peptides (5 mg/ml) were coupled to streptavidin-agarose beads for 1 hour at 4°C (Sigma), prior to bead saturation with D-biotin (1 mg/ml) for 1 hour at 4°C (Sigma). After 3 washes in lysis buffer, samples were combined with reducing sample buffer (New England Biolabs) and boiled, before separation on SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Anti-PTP1C (0.5 mg/ml) and anti-PTP1D (0.1 mg/ml) mAb were purchased form Transduction laboratories. Immunoblots were revealed using horseradish peroxidase-conjugated rabbit anti-mouse antiserum (Sigma) and ECL detection system (Amersham).

RBL cells were kindly obtained from Dr. Marc Daèron (Institut Curie, Paris) (Daèron et al. 1995. *Immunity* 3:1). RBL cells were transfected by electroporation using the 183.6 cDNA encoding p58.183, in the RSV-5.gpt expression vector (Wagtmann et al. 1995. *Immunity* 2:439). Stable transfectants were established by culture in the presence of xanthine (250 mg/ml), hypoxanthine (13.6 mg/ml) and mycophenolic acid (2 mg/ml). The cell surface expression of p58.183 was confirmed by immunofluorescence and flow cytometric analysis. Cells from a representative clone (T58.A) were stimulated for 3 minutes at 37°C in the presence or absence of 150 mM sodium pervanadate. Cells were then instantly lysed at 4°C in NP-40 lysis buffer, and immunoprecipitations were carried out using 10 mg/ml anti-CD3e or anti-GL183 mAb, and protein G sepharose beads (Pharmacia).

PTP1C/*PTP1D Binding.* PTP1C binding to phosphorylated p58.183 peptides was tested in human T and NK cell lysates. NK3.3 cells were lysed in NP-40 lysis buffer and adsorbed with unphosphorylated p58.183.1 as well as phosphorylated p58.183.1 and p58.183.2 peptides coupled to streptavidin-agarose beads. Affinity-bound proteins were resolved on 10% SDS-PAGE, transferred to nitrocellulose and probed with anti-PTP1C and anti-PTP1D mAb. For NKL cells (15 X 10^6 cells/sample) were prepared as described above and adsorbed with phosphorylated p58.183.1, p58.183.2 and CD3z.1 peptides bound to beads. The immunoblot was probed with anti-PTP1C mAb. For p58$^+$ T lymphocytes were prepared as described above and adsorbed with phosphorylated p58.183.1 and CD3ζ.1 peptides bound to

beads or directly resolved on SDS-PAGE. The immunoblot was probed with anti-PTP1C mAb.

RBL cells expressing the p58.183 molecule were stimulated or not with sodium pervanadate. NP-40-lysates were prepared and directly resolved on 8% SDS-PAGE under non-reducing conditions, or immunoprecipitated using anti-CD3ε (UCHT1, IgG1) or anti-p58.183 (GL183, IgG1) mAb. The immunoblot was probed with anti-PTP1C mAb.

The measurement of phosphorylated p58.183 peptide binding to recombinant GST-PTP1C (N+C)-SH2 fusion proteins and His-PTP1D was performed on a BIAcore using 500 nM recombinant protein in BIAcore HBS medium. The flow rate was kept constant at 10 ml/min. In this representative experiment, 100 and 150 RU of p58.183.1 and p58.183.2 phosphorylated peptides respectively were immobilized on streptavidin microchips. The regeneration buffer was BIAcore HBS medium supplemented with 10 mM NaOH (pH: 10). Results were expressed as corrected resonance units (CRU) corresponding to the raw RU values obtained for each sample subtracted from background RU value due to the injection medium.

To compare tyrosine-phosphorylated human and mouse NKIR binding to PTP1C and PTP1D, NP-40 lysates from A20 murine B lymphoma cells were adsorbed with either Ly49- or phosphorylated Ly49-beads. Samples were analyzed on SDS-PAGE and revealed with polyclonal rabbit anti-PTP1C and anti-PTP1D antibodies. WCL are whole cell lysates. Binding was inhibited by addition of 50 mM *p*-nitrophenyl phosphate (pNPP), a phosphotyrosine analog, to the lysates during adsorption.

A20 cell lysates were prepared as described above, and adsorption was performed using unphosphorylated or phosphorylated FcgRIIB1 ITIM peptides. Samples were analyzed on SDS-PAGE and revealed with polyclonal rabbit anti-PTP1C and anti-PTP1D antibodies. Binding was inhibited by addition of 50 mM *p*NPP to the lysates during adsorption.

NP-40 lysates were also prepared from Jurkat human T leukemia cells. Samples were resolved on 10% SDS-PAGE, transferred to nitrocellulose and probed respectively with anti-PTP1C and PTP1D mAbs.

Results

To directly test the possibility that human NKIR molecules contain two independent ITIM sequences, unphosphorylated and phosphorylated peptides surrounding the N-terminal Y_{303} (p58.183.1 peptide) and the C-terminal Y_{333} (p58.183.2 peptide) residues of p58.183 intracytoplasmic sequence were synthesized. These peptides were coupled to beads, and then used to adsorb lysates prepared from two distinct human NK cell lines, NK3.3 and NKL, as well as human $p58.183^+$ T cell lysates, prior to immunoblotting using anti-PTP1C mAb.

Each of the phosphorylated p58.183 peptides bind the PTP1C PTPase expressed in these cells. No binding was detected using unphosphorylated p58.183 peptides, demonstrating the requirement of p58 tyrosine phosphorylation for PTP1C recruitment. In addition, p58.183 immunoprecipitates prepared from p58.183 transfected cells contain PTP1C, confirming *in vivo* the association between PTP1C and phosphorylated NKIR. Importantly, this association was detectable only when p58.183 transfected cells were stimulated with pervanadate, an activator of PTK-dependent pathways (Donnadieu et al. 1994. *J Biol. Chem.* 269:32828), consistent with the binding of PTP1C to the tyrosine phosphorylated form of p58.183.

In addition to PTP1C, the family of SH2-tandem PTPase contains PTP1D (Syp, PTP2C, SH-PTP2) and the *Drosophilia* homologue corkscrew (csw). PTP1D is ubiquitously expressed and regulates PTK-dependent signaling mechanisms (Stone, R. L., and J. E. Dixon. 1994. *J. Biol. Chem.* 269:31323). Therefore, NK cell lysates adsorbed with unphosphorylated and phosphorylated p58.183 peptides were also probed for the binding of PTP1D. Each of the phosphorylated p58.183 peptides bind PTP1D, whereas no binding to unphosphorylated p58.183.1 was detected. The human NKIR p58 has been shown to be part of a multimeric non-covalent complex including FceRIg and CD3z (Bottino et al. 1994. *Eur. J. Immunol.* 24:2527). No binding of PTP1C and PTP1D to double tyrosine-phosphorylated CD3z.1 and FceRIg ITAM peptides can be detected, emphasizing the specificity of PTPases recruitment by tyrosine-phosphorylated human NKIR. Of note, no binding of other SH2-containing proteins, such as p56*lck* and p59*fyn* was detected under similar peptide adsorption and immunoblotting conditions (data not shown).

As with unphosphorylated p58.183.1 peptides, no binding to unphosphorylated p58.183.2 peptide was detected. The larger signal corresponding to PTP1D in this experiment is the consequence of a better recognition of PTP1D by anti-PTP1D mAb as compared to the recognition of PTP1C by anti-PTP1C mAb.

Real-time measurement of phosphorylated p58.183 binding to PTPase was then performed using surface plasmon resonance (SPR). Binding of phosphorylated p58.183.1 and phosphorylated p58.183.2 peptides to PTP1C was observed using recombinant GST-PTP1C-SH2 (N+C) fusion protein, confirming that phosphorylated NKIR directly binds the PTP1C PTPase via its SH2 domains. The N-terminal p58.183.1 phosphorylated peptides bind PTP1C with a higher affinity than the C-terminal p58.183.2 phosphorylated peptides, as assessed by calculation of the K_d values.

Table 1: Association and dissociation constants for the interaction of recombinant PTP1C, PTP1D, and ZAP proteins with human NKIR phosphorylated peptides.

Peptides	PTP1C GST-SH2 (N+C)				ZAP-70 GST-SH2 (N+C)				His-PTP1D
	CRU*	k_{on}	k_{off}	K_d	CRU	k_{on}	k_{off}	K_d	CRU
p58.183.1	2008	1.93 ± 0.70	1.40 ± 0.19	83.7 ± 0.9	1	—	—	—	182
p58.183.2	390	0.41 ± 0.02	1.97 ± 0.35	473.1 ± 56.6	8	—	—	—	42
CD3z.1	1	—	—	—	893	13.5 ± 0.6	0.75 ± 0.01	6.70 ± 0.04	8
		(10^{-4} M^{-1} s^{-1})	(10^{3} s^{-1})	(nM)		(10^{-4} M^{-1} s^{-1})	(10^{3} s^{-1})	(nM)	

100 RU of each phosphorylated peptides were immobilized on streptavidin microchips.

*: CRU were measured at the end of injection period.

k_{off} and k_{on} were calculated from three independent measurements using the BIAevaluation 2.0 software.

K_d was calculated from k_{off}/k_{on}.

Although the affinity of phosphorylated CD3z.1 for isolated ZAP-70 tandem SH2 domains, was higher than the affinities of p58.183 phosphorylated peptides for PTP1C tandem SH2 domains, the latter are consistent with the K_d values reported for the binding of PTP1D to PDGF receptor Y_{1009} phosphorylated peptides (Huyer et al. 1995. *Biochemistry* 34:1040). The binding of PTP1D to each of the phosphorylated p58.183 peptides was 4 to 6 times less efficient than PTP1C binding as measured by SPR Table 1). These data also confirmed a hierarchy in the binding affinity of p58.183 ITIM-like sequences, since the N-terminal p58.183.1 phosphorylated peptides bind more efficiently both PTP1C and PTP1D, than the C-terminal p58.183.2 phosphorylated peptides.

Consistent with our observation that members of the mouse Ly49 family also express an I/VxYxxL/V ITIM-like sequence, the recruitment of PTP1C and PTP1D PTPases by phosphorylated vs. unphosphorylated Ly49 intracytoplasmic motifs was tested.

Phosphorylated Ly49A peptides recruit PTP1C and PTP1D whereas no binding was detected with the unphosphorylated Ly49A peptides in A20 cell lysates. In these experiments, further confirmation of the requirement of tyrosine phosphorylation for Ly49A peptides binding to PTP1C was obtained by competition using a phosphotyrosine analog, *p*NPP. Thus, results obtained with Ly49A were similar to our observations with human NKIR and with FcgRIIB1 ITIM. However, compared to the binding observed with each of the p58.183 phosphorylated peptides, the bindings of PTP1C and PTP1D to phosphorylated Ly49A peptides are less efficient, as judged by the amount of PTPase adsorbed in Jurkat cell lysates. In these cells, no binding of Ly49A to PTP1C was observed, but this apparent discrepancy likely reflects the higher amount of PTP1C present in A20 cells as compared to Jurkat cells (data not shown). Indeed, the IC_{50} of phosphorylated Ly49A for competing phosphorylated p58.183.1 binding to PTP1C is in the same order of magnitude that the IC_{50} of phosphorylated p58.183.2 (Table 2), confirming that phosphorylated Ly49A does bind to PTP1C.

Table 2. IC_{50} values of various competitors for the interaction of PTP1C-SH2 (N+C) domains with phospho-p58.183.1 NKIR peptides.

100 RU of each phosphorylated peptides were immobilized on streptavidin microchips. IC_{50} were defined as the concentration of competitor ligand required to achieve a 50% reduction in the binding of GST-PTP1C-SH2 (N+C) to an immobilized phosphorylated p58.183.1 peptide. IC_{50} were calculated from competition assays using SPR: GST-PTP1C-SH2 (N+C) at 500 nM was preincubated with various concentrations (0 to 100 mM) of indicated free peptides, and injected over immobilized phosphorylated p58.183.1 surface.

competitor	IC_{50} (mM)
p58.183.1	no inhibition using 200 mM
phospho-p58.183.1	7.5
phospho-p58.183.2	65
phospho-CD3z.1	no inhibition using 200 mM
phospho-Ly49	90

Based on sequence homologies as well as on the recruitment of PTPases upon tyrosine phosphorylation, Applicant proposes that I/VxYxxL/V motifs expressed in both human and mouse NKIR intracytoplasmic domains can be defined as ITIM. Nevertheless, whereas phosphorylated human NKIR and FcgRIIB1 ITIM preferentially recruit PTP1C, our data indicate that the phosphorylated mouse NKIR ITIM binds PTP1D with a higher affinity than PTP1C. These observations also suggest a potential requirement of critical amino-acids N-terminal of the phosphotyrosine residue for recruitment of SH2 domains, in addition to the canonical pY+3 amino-acid (Songyang, Z., and L. C. Cantley. 1995. *TIBS* 20:470). Such a sequence recognition requirement has been previously documented for PTP1D SH2 binding to phosphotyrosine sequence, and includes a critical position for V at pY-2, consistent with our data (Huyer et al. 1995. *Biochemistry* 34:1040).

In contrast to ADCC-dependent and CD3/TCR-dependent transduction pathways, the signaling programs coupled to the engagement of MHC class I receptor are still unclear. The recruitment of PTPases by tyrosine phosphorylated ITIM present in NKIR intracytoplasmic sequence documents a signaling mechanism by which NKIR engagement can lead to the inhibition of NK and T cell activation programs. Similarly to the recruitment of PTPases by phosphorylated FcgRIIB1 ITIM, our results suggest that co-engagement of NKIR with an activatory receptor, i.e. CD3/TCR and ADCC receptor complexes in T and NK cells respectively, leads to the phosphorylation of intracytoplasmic NKIR ITIM, which in turn recruit PTP1C. The disregulation of activatory receptors, such as BCR, in PTP1C-deficient *mothheaten* mice indicates that dephosphorylation of signaling elements downstream of these receptors is central to the integrity of activatory responses (D'Ambrosio, et al. 1995. *Science* 268:293). It is noteworthy that the differentiation and function of NK cells isolated from *mothheaten* mice are defective (Koo et al. 1991. *J. Immunol.* 147:1194). Although PTP1C substrates are not identified, one attractive possibility is that the phosphorylated forms of SH2-tandem PTK, such as ZAP-70 and p72^{Syk}, might be dephosphorylated by PTP1C. Indeed in T cells, the recognition of altered peptide ligands by the CD3/TCR complex induces the recruitment of ZAP-70 by CD3z ITAM without ZAP-70 phosphorylation, leading to the generation of abortive signals (Madrenas et al. 1995. *Science* 267:515; de Magistris et al. 1992. *Cell* 68:625). It is thus conceivable that a similar strategy might also have been used by NKIR to inhibit T and NK cell activation programs. In this regard, it has been recently described that the recognition of target cells protected from NK cell lysis by the surface expression of HLA-B alleles leads to an inhibition of phosphatidyl inositol 4,5 biphosphate hydrolysis, resulting in the prevention of intracytoplasmic calcium mobilization (Kaufman et al. 1995. *Proc. Natl. Acad. Sci. USA* 92:6484). Likewise, an absence of phosphatidyl inositol 4,5 biphosphate hydrolysis has been described after engagement of the CD3/TCR complex by altered peptide ligands (de Magistris et al. 1992. *Cell* 68:625).

Another possibility is that members of the Janus PTK (JAK) family might be targets of the PTPase recruited by NKIR. The involvement of JAK PTK in NK cell activation programs has not been reported yet, but $JAK3^{-/-}$ mutant mice lack NK cells and PTP1C is involved in the down-regulation of JAK1, JAK2 and TYK2 function (Park et al 1995. *I mmunity* 3:771; Yetter et al. 1995. *J. Biol. Chem.* 270:18179).

It remains to be elucidated whether the binding of phosphorylated NKIR leads to the activation of PTPase catalytic activity, as has been described for phosphorylated FcgRIIB1 ITIM and CD22 (D'Ambrosio et al. 1995. *Science* 268:293; Doody et al. 1995. *Science* 269:242; Campbell, M. A., and N. R. Klinman. 1995. *Eur. J. Immunol.* 25:1573). In this regard, simultaneous occupancy of PTP1C or PTP1D tandem SH2 domains result in a more potent stimulation of PTPase activity than the occupancy of only one SH2 domain (Pluskey et al. 1995 *J. Biol. Chem.* 270:2897). Although each of the p58.183 phosphorylated peptides can bind PTP1C SH2 domains, each phosphorylated NKIR ITIM mainly binds to the same PTP1C-SH2 domain, and do not support the potentiation of PTPase activity due to the double SH2 occupancy. Irrespective of the effect of phosphorylated NKIR on PTPase function, it is also possible that the recruited PTPases act as docking molecules for recruiting and/or activating in turn other effector/adaptor molecules, as has been shown for PTP1D (Li, et al. 1994. *Mol. Cell. Biol.* 14:509).

The molecular dissection of natural cytotoxicity programs led to the discovery of NKIR. Although mouse NKIR are lectins and human NKIR contain Ig-like domains, both receptors bind MHC class I, and transduce inhibition of NK and T cell activation programs upon engagement. Our data show that despite their major structural difference, human and mouse NKIR recruit, upon intracytoplasmic tyrosine phosphorylation, the SH2-tandem PTPases PTP1C and PTP1D. This analogy between mouse lectin NKIR and human Ig-like NKIR suggests that both types of receptors share common transduction pathways, and document a striking convergent evolution for the recognition of MHC class I by T and NK lymphocytes, illustrating the importance of MHC class I quality control.

Example 10. This example illustrates that negative FCγRIIB1 signaling is mediated by inhibition of CD19 tyrosine phosphorylation and association with PI 3-kinase

The B-cell receptor for IgG, FcgRIIB1, is a potent transducer of negative signals which block B cell activation and the immune response. Co-ligation of FcgRIIB1 and B lymphocyte antigen receptor (BCR) causes premature termination of phosphoinositide hydrolysis and Ca^{2+} mobilization, blocking BCR mediated proliferative responses. Previous

studies have revealed that this inhibitory signal is mediated by phosphorylation of FcgRIIB1 Tyr^{309}, which recruits and activates the protein tyrosine phosphatase PTP1C. However, the target(s) of PTP1C is unknown. As is disclosed herein, FcgRIIB1 inhibition of BCR signalling is mediated by dephosphorylation of CD19, a BCR coreceptor. Dephosphorylation of CD19 leads to diminished association of CD19 with PI-3 kinase and subsequent failed BCR mediated Ca^{2+} mobilization. The results define a molecular mechanism by which FcgRIIB co-engagement with BCR inhibits humoral immune responses.

Ligation of the antigen receptor on B lymphocytes leads to rapid tyrosine phosphorylation of the B cell receptor (BCR) subunits Iga and Igb, and the corecptor CD19 (D. A. Tuveson, et al. *Science* 260, 986-989 (1993) N. J. Chalupny, et al., *EMBO Journal* 12, 2691-2696 (1993)). Phosphorylation of Iga (tyr^{182} & 193) and Igb (tyr^{195} & 206) (H. Flaswinkel, M. Reth, *EMBO Journal* 13, 83-89 (1994); D. Choquet, et al., *Journal of Biological Chemistry* 269, 6491-6497 (1994)) leads to recruitment and activation of Src and Syk family kinases that bind BCR via their SH2 domains (A. L. Burkhardt et al. *Proceedings of the National Academy of Sciences USA* 88, 7410-7414 (1991); T. Yamada, et al.. *European Journal of Biochemistry* 213, 455-459 (1993); S. A. Johnson, et al., *Journal of Immunology* 155, 4596-4603 (1995)). In addition, BCR ligation also results in activation of $p21^{ras}$ (A. E. Harwood, J. C. Cambier, *Journal of Immunology* 151, 4513-4522 (1993)), PLC-g1 and 2 (K. M. Coggeshall et al. *Proceedings of the National Academy of Sciences USA* 89, 5660-5664 (1992)), and Ca^{2+} mobilization. CD19 phosphorylation at tyrosine residues 484 and/or 515 initiates recruitment and activation of PI-3 kinase, and phosphorylation of tyrosine 393 may recruit the multifunctional oncoprotein VAV (D. A. Tuveson, et al. *Science* 260, 986-989 (1993)).

Recruitment and subsequent activation of these effectors leads to a variety of responses including increased expression of cell surface molecules, including: MHC class II and B7.2 (CD86) that function in T-cell/B-cell collaboration, as well as in apoptosis, proliferation, and differentiation.

Coligation of the B cell's low affinity receptor for IgG immunoglobulin constant region (FcgRIIB1) with the antigen receptor leads to abortive BCR signalling, blocking biologic responses (P. L. Chan, N. R. S. C. Sinclair, *Immunology* 21, 967-981 (1971); N. E. Phillips, D. C. Parker, *Journal of Immunology* 130, 602-606 (1983)). Previous studies have indicated that the inhibitory function of FcgRIIB is mediated by a 13 amino acid segment containing a tyrosine residue that is phosphorylated upon receptor coligation (S. Amigorena, et al., *Science* 256, 1808-1812 (1992); W. H. Fridman, et al., *Immunological Reviews* 125, 49-76 (1992); T. Muta, et al., *Nature* 368, 70-74 (1994)). This phosphorylation results in binding and activation of the SH2-containing tyrosine phosphatase PTP1C (a.k.a. HCP, SHP,

and SHPTP-1) (D. D'Ambrosio, et al., *Science* 268, 293-297 (1995)), whose function in the BCR signalling cascade is unknown.

Materials and Methods

Antibodies. Antibodies used in this study are all rabbit polyclonals. The immunogens for CD19 and Iga were the complete cytoplasmic domains, lyn (residues 1-131), Syk (linker region as described [C. G. Couture, et al., Mol. Cell Biol.13, 5249 (1994)]), p85 (N-terminal SH3 domain). Anti-VAV was from Santa Cruz Biotechnology, Inc., anti-p85 from Upstate Biotechnology, Inc. (used for western blotting), and anti-phosphotyrosine (Ab-2) from Oncogene Science. For immunoprecipitations, affinity purified antibodies (except for anti-syk) were coupled to CNBr-Sepharose at 1 mg/ml, and 20 µg. Syk immunoprecipitations were accomplished using 20 µl crude serum and 10 µl protein-A Sepharose (Pharmacia). Western blotting was accomplished with a 1:1000 dilution of primary antibody, and peroxidase-conjugated protein A (Amersham) or rat anti-mouse IgG1 (Zymed) for phosphotyrosine.

CD19 construct. The human CD19 cDNA was isolated from pMT2 (gift of Thomas Tedder, Duke University) by EcoR1 digest, and subcloned into pSK Bluescript (Stratagene). The CD19 cDNA was then removed from pSK with Hind III/Cla I restriction digest and cloned into pLNCX (Williams, Fred Hutchinsen Cancer Center, Seatle, WA). This construct was electroporated into GP+E/NIH3T3 cells and cell supernatant collected at 72 hours. After a 12 hour incubation in tunicamycin (1µg/ml), GP+E/NIH3T3 cells were infected with pLNCX/hCD19 retrovirus by incubating with the cell supernatant collected above. Following G418 (Gibco-BRL) selection (1 mg/ml), virus expressing clones were selected. Virus collected from these cells was then used to infect J558Lµm13 myeloma cells. Human CD19 positive J558Lµm3 cells were obtained after G418 selection (1 mg/ml) by cell sorting of the bulk infected population with a mouse monoclonal to human CD19 (Caltag Laboratory, San Francisco, CA).

Tyrosine phosphorylation experiments. Cells (10^8/ml) were left unstimulated or stimulated with F(ab')2 (12 µg/ml) or intact (20 µg/ml) rabbit anti-mIg, pelleted in a picofuge and stimulating antibody removed, then lysed in 1% NP-40 lysis buffer [1% NP-40, 150 mM NaCl, 10 mM tris-HCl (pH 7.4), 1.0 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 0.4 mM EDTA, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 2 µg/ml each of aprotinin, leupeptin, and a-1-antitrypsin] and spun 5 min at 14,000 rpm in an Eppendorf microfuge to remove detergent-insoluble material. To reduce nonspecific bands,

SDS was added to the cleared lysates (0.5% final), which were then boiled 5 minutes and diluted 1 to 5 in lysis buffer. Lysates were then incubated 15 minutes with antibodies to the signalling molecules indicated, washed rapidly 3x in lysis buffer, then eluted with SDS reducing sample buffer, fractionated by SDS-PAGE, and transferred to immobilon-P membranes (Millipore). The membranes were blocked with 5% bovine serum albumin, immunoblotted sequentially with anti-phosphotyrosine and anti-effector antibodies, and developed with an enhanced chemiluminescence (ECL) detection system (Amersham).

To measure the effect of FcgRIIB1 coligation on BCR mediated tyrosine phosphorylation. A20 and IIA1.6 (an FcgRIIB1 negative variant of A20) B lymphoma cells were cultured without stimulus or were stimulated with intact or $F(ab')_2$ fragments of rabbit antibody to mouse immunoglobulin. After sequential immunoblotting for phosphotyrosine and effector, the images were scanned, and the pixel density of the anti-phosphotyrosine signal and anti-effector signal determined. The values were normalized for amount of effector present.

p85/CD19 association. A20 cells were cultured without stimulus or were stimulated with intact or $F(ab')_2$ rabbit anti-mouse Ig antibody for 2 min. The cells were lysed and CD19 or p85 immunoprecipitated as described. The immunoprecipitates were washed, eluted with SDS reducing sample buffer, fractionated by SDS-PAGE on a 10% gel, and transferred to immobilon-P membranes. The membranes were sequentially immunoblotted with anti-CD19, and anti-p85 as above. The coprecipitation of PI 3-kinase activity with CD19 was assessed from A20 cells that were stimulated and lysed as above. Immunoprecipitates were washed, assayed for PI 3-kinase activity using PI as the substrate, and reaction products resolved by thin-layer chromatography. The incorporation of $^{32}PO_4$ into phosphatidylinositol phosphate (PIP) was quantitated using a Phosphorimager.

Results

In order to define the target(s) of PTP1C in the antigen receptor signalling pathway, Applicant compared substrate tyrosine phosphorylation following crosslinking of BCR alone and cocrosslinking FcgRIIB1 with BCR. The FcgRIIB1 positive murine B lymphoma cell line A20 and its FcgRIIB1 negative variant IIA1.6 were stimulated with either intact (I) or $F(ab')_2$ rabbit anti-mouse immunoglobulin, and the spectra of proteins phosphorylated on tyrosine compared by anti-phosphotyrosine immunoblotting of whole cell lysates. Among prominent substrates phosphorylated upon stimulation of BCR alone, only one major species was not seen upon BCR-FcgRIIB1 coligation. This species, which has an apparent molecular mass of ~115 kDa, migrated at a position corresponding to murine CD19. Phosphorylation

of FcgRIIB1 and an unidentified species at 160 kDa were seen only when FcgRIIB1 and BCR were crosslinked. Neither failed phosphorylation of CD19 nor phosphorylation of p160 and FcgRIIB1 were seen when FcgRIIB1 negative cells were stimulated with intact anti-receptor antibody. These data suggest that among dominant substrates that are tyrosine phosphorylated upon BCR ligation, CD19 may be a substrate for PTP1C.

The possibility that CD19 is prematurely dephosphorylated upon FcgRIIB1 coligation with BCR was confirmed by anti-phosphotyrosine immunoblotting of SDS-PAGE fractionated immunoprecipitates from stimulated B cells. Among substrates analyzed, including CD19, Iga, VAV, Lyn and Syk, CD19 was phosphorylated most rapidly upon BCR ligation and was immediately dephosphorylated upon FcgRIIB1 coligation. CD19 exhibited significantly reduced levels of tyrosine phosphorylation (73%) even at the earliest time point tested (15 sec). Reduced tyrosine phosphorylation of Iga was apparent only at 45 seconds and decreased to background levels by 120 seconds. Slight decreases in the tyrosine phosphorylation state of Syk were detected at 120 seconds and 300 seconds. but no differences were detected in Lyn or VAV phosphorylation. Consistent with their lack of FcgRIIB1 expression, stimulation of IIA1.6 with either intact or $F(ab')_2$ anti-receptor antibodies induced equivalent levels and duration of protein tyrosine phosphorylation of these substrates.

Previous studies have shown that phosphorylation of tyrosine residue(s) 484 and/or 515 in CD19 leads to binding of PI-3 kinase via the SH2 domains of the PI-3 kinase p85 subunit (D. A. Tuveson et al. *Science* 260, 986-989 (1993)). To investigate whether FcgRIIB1 mediated decreases in CD19 phosphorylation prevented or terminated its association with PI 3-kinase, CD19 and PI 3-kinase were immunoprecipitated from NP-40 lysates of intact or $F(ab')_2$ stimulated A20 cells. The immunoprecipitates were fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF membrane and blotted sequentially with anti-CD19 and anti-p85. In agreement with previously published results (D. A. Tuveson, *supra.*), that the amount of p85 associated with CD19 greatly increased when cells were stimulated with $F(ab')_2$ anti-receptor antibodies. In contrast, levels of p85 associated with CD19 remained near basal levels when cells were stimulated with intact antibody that cocrosslinked FcgRIIB1 and BCR. This increased association was demonstrable by precipitation with either anti-CD19 or anti-p85. Thus FcgRIIB1 coligation with BCR caused CD19 dephosphorylation preventing its recruitment of p85 detectable based on immunoblotting.

To further assess whether the decreased phosphorylation of CD19 results in decreased recruitment of PI 3-kinase activity to the receptor-coreceptor complex, PI 3-kinase assays were performed on CD19 immunoprecipitates from unstimulated A20 cells, or A20 cells

stimulated with either F(ab')2 or intact rabbit anti-mouse Ig. CD19 isolated from unstimulated cells contained levels of PI 3-kinase activity that were only slightly above the background of the assay. However, following BCR stimulation (F(ab')2), the amount of PI 3-kinase activity associated with CD19 was increased more than 19-fold. Importantly, in addition to the effects on CD19 tyrosine phosphorylation, coligation of FcgRIIB1 with BCR reduced CD19 association with PI-3 kinase activity 75%. Control experiments with the FcgRIIB-negative cell line IIA1.6 confirmed the Fc receptor dependence of this decreased association.

A well documented effect of FcgRIIB1 coligation with BCR is premature termination of BCR mediated extracellular calcium influx (see Figure 2) (H. A. Wilson, et al., *Journal of Immunology* 138, 1712-1718 (1987); D. Choquet, et al., *Journal of Cell Biology* 121, 355-363 (1993)). Taken together, this finding and those described above suggest that abortive Ca^{2+} mobilization may result from failed PI 3-K activation. PI 3-kinase activity has been implicated in Ca^{2+} mobilization in some, but not all cell types. To determine whether PI 3-kinase is involved in BCR mediated Ca^{2+} mobilization, Applicant assessed the effect of the PI 3-kinase inhibitors, Wortmannin (H. Yano, et al., *Journal of Biological Chemistry* 268, 25846-25856 (1993); T. Okada et al. *Journal of Biological Chemistry* 269, (1994)) and Ly294002 (C. J. Vlahos et al. *Journal of Biological Chemistry* 269, 5241-5248 (1994)), on BCR mediated Ca^{2+} mobilization. As shown in Figure 2, 10 nM Wortmannin inhibited BCR mediated Ca^{2+} mobilization significantly. Similar results were seen with Ly294002. Unlike FcgRIIB1 cocrosslinking, Wortmannin inhibited both immediate and prolonged Ca^{2+} responses. This may reflect the time lag imposed by the necessity to recruit and activate FcgRIIB1 and PTP1C to achieve negative signalling upon FcgRIIB1-BCR coligation. The above results are consistent with a critical role for CD19 phosphorylation and PI 3-kinase translocation in BCR mediated Ca^{2+} mobilization and its disruption by FcgRIIB1 coligation.

To address the relationship of PI 3-kinase and Ca^{2+} mobilization more directly, BCR mediated Ca^{2+} mobilization was analyzed in a plasmacytoma cell line (J558Lμm3CD45+) that expresses an exogenous B cell antigen receptor and CD45, but no CD19 as judged by immunoblotting. Human CD19+ variants of this cell were produced by retroviral transformation (Figure 3). Ligation of the BCR in the $CD19^-$ cell line resulted in Ca^{2+} mobilization, but only an initial flux was observed. In contrast, when CD19 was expressed, initial and prolonged Ca^{2+} mobilization was observed, reminiscent of normal B cells and A20 cells. Therefore, as observed when FcgRIIB1 is coligated to BCR, lack of CD19 function results in a loss of prolonged Ca^{2+} mobilization.

These results indicate that CD19 mediated recruitment and activation of PI 3-kinase is necessary for BCR mediated activation of extracellular Ca^{2+} influx. Although the molecular basis of PI 3-kinase modulation of this response is unclear, recent observations that

polyphosphorylated inositol lipid products of PI 3-kinase bind SH2 domains raise the possibility that PI 3-kinase activation leads to PLCg translocation to the plasma membrane where its substrate resides (L. E. Rameh, C. S. Chen, L. C. Cantley, *Cell* 83, 821-830 (1995)). This may facilitate polyphosphoinositide hydrolysis and, via IP_3 release, calcium mobilization.

Recent genetic manipulation of CD19 expression using transgenic and gene ablated animals have allowed studies which demonstrate a critical role for CD19 in B cell development and immune responses (L. J. Zhou, et al., *Molecular and Cellular Biology* 14, 3884-3894 (1994)R. C. Rickert, K. Rajewsky, J. Roes, *Nature* 376, 352-355 (1995); P. Engel, et al., *Immunity* 3, 39-50 (1995); S. Sato, D. A. Steeber, T. F. Tedder, *Proceedings of the National Academy of Sciences USA* 92, 11558-11562 (1995)). Overexpression of CD19 leads to impaired B cell development and reduced B cell numbers in the periphery (L. J. Zhou, et al., *Molecular and Cellular Biology* 14, 3884-3894 (1994)). Applicant hypothesizes that overexpression of CD19 may lead to enhanced BCR-CD19 signalling in response to selecting antigen. This heightened signalling, mediated by increased PI 3-kinase activation may result in deletion rather than selection of B cells and be manifested as defective B cell development. Studies of the immune responsiveness of B cells from gene ablated mice have revealed that CD19 plays a critical role in BCR mediated activation. as well as germinal center formation. Thus, CD19 may enhance sensitivity to antigen by enhancing activation of PI 3-kinase and other effectors. Consistent with this hypothesis, cocrosslinking of CD19/CD21 with BCR greatly enhances B cell activation (R. H. Carter, D. T. Fearon, *Science* 256, 105-107 (1992); L. D. Shultz, et al., *Cell* 73, 1445-1454 (1993)). Based on these findings one would predict that if CD19 is the target of FcgRIIB1/PTP1C mediated signalling, the consequences of FcgRIIB1-BCR cocrosslinking in normal B cells should be similar to those of BCR crosslinking in $CD19^{-/-}$ B cells. This is in fact the case; stimulation in either situation leads to impaired proliferation and antibody production (P. L. Chan, N. R. S. C. Sinclair, *Immunology* 21, 967-981 (1971); N. E. Phillips, D. C. Parker, *Journal of Immunology* 130, 602-606 (1983) P. Engel, et al., *Immunity* 3, 39-50 (1995); S. Sato, D. A. Steeber, T. F. Tedder, *Proceedings of the National Academy of Sciences USA* 92, 11558-11562 (1995)). Finally, also consistent with a role for PTP1C as a negative regulator of CD19 signalling are certain phenotypic traits of motheaten ($PTP1C^{-/-}$) mice. These mice exhibit increased levels of circulating immunoglobulin as seen in $CD19^{+/+}$ mice relative to $CD19^{-/-}$ mice (L. D. Shultz, et al., *Cell* 73, 1445-1454 (1993)). Lack of PTP1C in motheaten mice may enhance CD19 signalling, and increase circulating immunoglobulin levels. The studies presented elucidate a novel molecular mechanism for integration of positive and negative transmembrane signals. The dynamic interplay of these pathways clearly plays an important role in immune regulation.

Example 11. This example describes the use of alanine scanning mutagenesis to detect critical ITIM residues

For these experiments IIA1.6 cells, Fcγ RIIB1 negative mutants of A20 B cell lymphoma cells, were reconstituted with FcγRIIB1 which had been subjected to alanine scanning mutagenesis using standard techniques (Cunningham and Wells (1989) *Science* 244:1081-1085). Cells expressing FcγRIIB1 with alanine substitutions at various sites were assayed for their ability to inhibit B cell activation following co-ligation of the receptors with the BCR was assessed. B cell activation was assessed by measuring changes in intracellular Ca^{2+} after stimulation of cells with antibody. Cells were loaded with Indo 1AM as described in the previous examples. Indo-loaded cells were then stimulated with 20 ug/ml intact anti-mIg antibodies and the Ca2+ responses were recorded by flow cytometry as described above. The ability of a FcγRIIB1 receptor ITIM replaced with an alanine at each of the amino acids shown to inhibit Ca^{2+} fulx was scored with a + (i.e. lack of an effect of the mutation) and the inability of a mutated ITIM to influence Ca^{2+} flux was scored with a - (i.e. the mutation resulted in a loss of function of the ITIM). The results are shown in Table 3 and indicate that in addition to the critical tyrosine residue, the amino acids at the-1, -2 and +3 positions relative to the tyrosine are critical for ITIM inhibitory function.

Table 3. Alanine Scanning Mutagenesis of ITIM Sequence

position	T	I	T	Y	S	L	L	K	H
effect	+	-	-	-	+	+	-	+	+

Example 12. This example shows that the IgE mediated activation of bone marrow derived basophils is inhibited by FcgR signaling. .

Murine bone marrow derived mast cells were harvested from mouse femurs using standard techniques. Cells were cultured in vitro with IL-3 and c-kit ligand to enrich for the presence of mast cells. The resulting cells were greater than 95% mast cells based on FcεR expression and functional criteria (release of seratonin upon stimulation). These bone marrow mast cells expressed both FcγRII and FcεR. Cells were loaded with Indo 1A as described in the previous Examples. Cells were next incubated with monovalent IgE antibody to occupy surface IgεR without activating the cells. Cells were next incubated with control or $F(ab')_2$ fragments of rabbit anti-mouse Ig, which recognizes both IgG and IgE. Ca^{2+} flux was measured using flow cytometry as described above. The use of $F(ab')_2$ fragements to crosslink FcεR resulted in an increase in intracellular free Ca^{2+}. The use of control rabbit

anti-mouse Ig, which was capable of binding FcgRII, resulting in signal transduction through both FcεR and FcγRII resulted in an inhibition of Ca^{2+} flux. This Example shows that the FcgRII is capable of mediating a downregulatory response in mast cells which is similar to that which it mediates in B cells. In addition, the same expreriments were performed in motheaten mice, which are deficient in PTP1C. In bone marrow mast cells from these animals, no inhibition of Ca^{2+} flux was seen when FceR and FcgRII were crosslinked. Thus demonstrating the importance of PTP1C in the downregulatory response.

While various embodiments of the present invention have been described in detail, it is apparent that modifications and adaptations of those embodiments will occur to those skilled in the art. It is to be expressly understood, however, that such modifications and adaptations are within the scope of the present invention, as set forth in the following claims.

All of the above-cited references and publications are hereby incorporated by reference.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What is claimed is:

1. A peptidyl compound comprising an ITIM motif represented in the general formula:

$$(I/V)X_1YX_2X_3(L/V)$$

wherein, I represents isoleucine or a mimetope thereof, V represents valine or a mimetope thereof, each of X_1, X_2 and X_3 independently represents any amino acid or mimetope thereof, Y represents a tyrosine, phosphotyrosine or a mimetope thereof, and L represents leucine or mimetope thereof.

2. The compound of claim 1, wherein X_1 represents a Gly, Ala, Val, Ile, Leu, Ser, Thr, Cys, Glu, Asp, Lys, Arg or His; X_2 represents a Gly, Ala, Val, Ile, Leu, Ser, Cys or Thr; and X_3 represents a Gly, Ala, Val, Ile, Leu, Ser, Cys, Thr, Met, Asn, Gln, Glu or Asp.

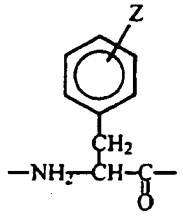
3. The compound of claim 1, wherein X_1 represents a Val, Ile, Leu, Ser, Thr, Asp or His; X_2 represents an Ala, Val, Ser or Thr; and X_3 represents a Leu, Ile, Thr, Met, Gln, Glu or Asp.

4. The compound of claim 1, wherein the ITIM motif is represented by one of ITYSLL, VTYAQL, IVYTEL, VTYTQL, IVYTEL, VTYAQL, ILYTEL, VTYSMV, VTYTTL, VTYSTV, VIYSDL, or ITYAEL.

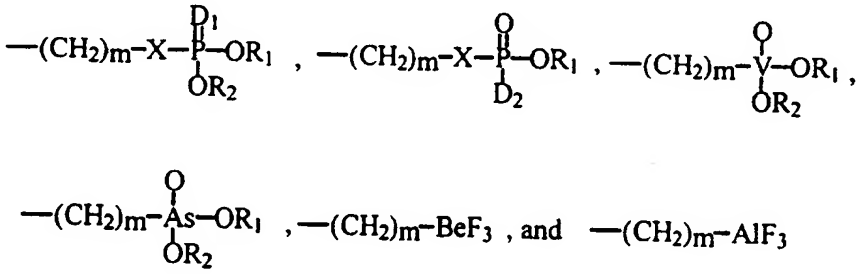
5. The compound of claim 4, wherein the ITIM motif is represented by one of GAENTITYSLLMH, TAENTITYSLLKH, EQDPQEVTYAQLN, TPPTDIIVYTELP, EQDPQEVTYTQLN, TPPTDIIVYTELP, EQDPEEVTYAQLD, TPPTDTILYTELP, MSEQEVTYSMVRF, MSEQEVTYTTLRF, MSEQEVTYSTVRF, or MDNQGVIYSDLNL.

6. The compound of claim 1, wherein Y is a non-hydrolyzable phosphotyrosine analog.

7. The compound of claim 1, wherein Y is represented by the general formula:



,where Z is selected from a group consisting of



where m is zero or an integer in the range of 1 to 6; X is absent or represents O, S, or N; D_1 represents O or S; D_2 represents N_3, SH_2, NH_2, or NO_2; and R_1 and R_2 each independently represent hydrogen, a lower alkyl, or a pharmaceutically acceptable salt, or R_1 and R_2 taken together with the O-P-O, O-V-O or O-As-O atoms to which they are attached complete a heterocyclic ring having from 5 to 8 atoms in the ring structure.

8. The compound of claim 1, wherein at least a portion of the compound is derived from naturally occurring amino acid residues.

9. The compound of claim 1, which compound is a peptidomimetic.

10. The compound of claim 9, which compound is a peptidomimetic is selected from the group consisting of a retro-inverso peptidomimetic, a retro-enatio peptidomimetic, a trans-olefin peptidomimetic and a phosphonate peptidomimetic.

11. The compound of claim 1, which compound has a molecular weight in the range of 750 to 7500 daltons.

12. The compound of claim 1, corresponding to a peptide being from 6 to 25 amino acids in length.

13. The compound of claim 1, wherein the ITIM motif is covalently linked by one or more peptide bonds with no more than 50 amino acid residues, in addition to the ITIM motif, which are identical to polypeptide sequences immediately contigous with the ITIM motif in a protein in which the ITIM motif naturally occurs.

14. The compound of claim 1, which compound is a fusion protein comprising the ITIM motif and a second amino acid sequence.

15. The compound of claim 1, which compound binds to protein tyrosine phosphatase 1C (PTP1C) and activates the phosphatase activity of the PTP1C.

16. The compound of claim 1, wherein the ITIM motif binds to a cellular component selected from the group consisting of a PTP1C, PTP1D, p160 and p70.

17. The compound of claim 16, which binding inhibits a signal transduction acitivity of the cellular component.

18. The compound of claim 16, which binding activates a signal transduction acitivity of the cellular component.

19. The compound of claim 1, wherein the ITIM motif binds to SH2 domains of a signal transduction protein.

20. The compound of claim 1, which compound regulates hematopoietic cell function by altering the enzymatic activity of an ITIM-binding protein.

21. The compound of claim 1, which compound regulates hematopoietic cell function by altering the signal transduction pathway of an MIRR.

22. The compound of claim 1, which compound competitively inhibits binding of a PTPase with the cytoplasmic domain of CD22, NKIRp58, Ly49, or FcγR.

23. A pharmaceutical preparation comprising the compound of claim 1, in a physiologically acceptable carrier, in a therapeutically-effective amount useful for modulating an intracellular signaling pathway in cells in of an animal being treated.

24. An ITIM mimetic which binds to an SH2 domain of protein tyrosine phosphatase 1C (PTP1C), activates the PTP1C phosphatase activity, and competitively inhibits binding of with an ITIM motif of any one or more of CD22, NKIRp58, Ly49 and Fcγ receptors.

25. An isolated ITIM peptide, or mimetic thereof, comprising an ITIM motif represented by the general formula (I/V)X_1YX$_2$X$_3$(L/V) (SEQ ID No:1) and corresponding to an ITIM sequence in a protein which binds to an SH2 domain of a protein tyrosine phosphatase, wherein, I represents isoleucine or a mimetope thereof, V represents valine or a mimetope thereof, each of X_1, X_2 and X_3 independently represents any amino acid or mimetope thereof, Y represents a tyrosine, phosphotyrosine or a mimetope thereof, and L represents leucine or mimetope thereof.

26. A soluble peptide fragment of a negative regulatory receptor selected from the group consisting of CD22, NKIRp58, Ly49 and Fcγ receptors, which peptide includes an ITIM motif.

27. The peptide of claims 26, which peptide has a molecular weight in the range of 750 to 7500 daltons.

28. The peptide of claims 26, which peptide binds to an SH2 domain of protein tyrosine phosphatase 1C (PTP1C).

29. The peptide of claims 28, wherein the binding of the peptide to PTP1C allosterically regulates a tyrosine phosphatase activity of the PTP1C.

30. The compound of claim 26, wherein the receptor is one of FcγRI, FcγRII, FcγRIII, Ly49, NKIRp58.

31. The compound of claim 26, wherein the receptor is FcγRIIB1.

32. A method for modulating an intracellular signaling pathway in a cell comprising altering an activity of an ITIM-binding protein involved in the intracellular signaling pathway by contacting the cell with a compound comprising an ITIM motif represented by the general formula:

$$(I/V)X_1YX_2X_3(L/V) \text{ (SEQ ID No:1)}$$

wherein, I represents isoleucine or a mimetope thereof, V represents valine or a mimetope thereof, each of X_1, X_2 and X_3 independently represents any amino acid or mimetope thereof, Y represents a tyrosine, phosphotyrosine or a mimetope thereof, and L represents leucine or mimetope thereof.

33. A method for regulating hematopoietic cell function, comprising contacting a hematopoietic cell with an effective amount of a regulatory reagent capable of altering the activity of a regulatory molecule selected from the group consisting of PTP1C, PTP1D, ITIM-p160 and ITIM-p70.

34. The method of claim 33, wherein the regulatory agent comprises an ITIM mimetic.

35. The method of claim 33, wherein the ITIM mimetic is a peptide or peptidomimetic having an ITIM motif represented in the general formula:

$$(I/V)X_1YX_2X_3(L/V) \text{ (SEQ ID No:1)}$$

wherein, I represents isoleucine or a mimetope thereof, V represents valine or a mimetope thereof, each of X_1, X_2 and X_3 independently represents any amino acid or mimetope thereof, Y represents a tyrosine, phosphotyrosine or a mimetope thereof, and L represents leucine or mimetope thereof.

36. The method of claim 33, wherein the regulatory agent is a bispecific antibody capable of crosslinking a stimulatory receptor protein with an inhibitory ITIM-containing receptor protein.

37. The method of claim 33, wherein the hematopoietic cell function is selected from the group consisting of antibody responses, blood clotting initiation, inflammatory responses, release of cytokines or mediators, or cytotoxicity

38. The method of claim 33, wherein the hematopoietic cell function is selected from the group consisting of inflammatory responses or antibody responses..

39. The method of claim 33, wherein the method regulates hematopoietic cell function by inhibiting the hematopoietic cell function.

40. The method of claim 33, wherein the method regulates the production and/or release of molecules selected from the group consisting of antibodies, cytokines, or inflammatory mediators.

41. The method of claim 33, wherein the method regulates hematopoietic cell function by inhibiting the activity of an MIRR.

42. The method of claim 33, wherein the regulatory reagent stimulates the specific phosphatase activity of PTP1C.

43. The method of claim 33, wherein the method regulates a hematopoietic cell function involved in a disease selected from the group consisting of an immunoproliferative diseases, immunodeficiency diseases, cancers, autoimmune diseases, infectious diseases and allergic responses.

44. The method of claim 33, wherein the method protects an animal from a disease selected from the group consisting of a disease involving autoantibody production, a disease involving FcεR induced degranulation or a disease involving ADCC.

45. The method of claim 33, wherein the cell is a B cell

46. The method of claim 45, wherein the treatment inhibits antibody production.

47. The method of claim 33, wherein the cell is one of a mast cell or basophil.

48. The method of claim 47, wherein the treatment inhibits an allergic response.

49. The method of claim 33, wherein the cell is an NK cell.

50. The method of calim 33, wherein the cell is treated ex vivo.

51. The method of claim 33, wherein the cell is treated in vivo.

52. A method for treating an autoimmune disorder comprising contacting hematopoietic cells of a patient with an amount of an ITIM mimetic capable of altering the activity of a regulatory molecule selected from the group consisting of PTP1C, PTP1D, ITIM-p160 and ITIM-p70.

53. A method for treating an allergic reaction disorder comprising contacting hematopoietic cells of a patient with an amount of an ITIM mimetic capable of altering the activity of a regulatory molecule selected from the group consisting of PTP1C, PTP1D, ITIM-p160 and ITIM-p70.

54. A method for treating an allergic reaction disorder comprising contacting hematopoietic cells of a patient with an amount of a bispecific antibody capable of crosslinking an Fcε receptor with an Fcγ receptor.

55. A method of identifying agents capable of altering the function of an SH2 containing protein comprising:

(a) forming a reaction mixture including

(i) a polypeptide comprising an ITIM motif

(ii) a target protein including at least one SH2 domain which specifically binds the ITIM motif by the SH2 domain, and

(iii) a test agent;

(b) detecting interaction of the target protein with the ITIM motif,

wherein a statistically significant change in the interaction of the target protein and ITIM polypeptide in the presence of the test agent, relative to the level of interaction in the absence of the test agent, indicates an activity of the test agent for altering a signal transduction activity of the SH2-containing target protein.

56. The method of claim 55, wherein one or more of the above components is recombinantly expressed.

57. The method of claim 57, wherein the SH2 containing target is a PTPase.

58. The method of claim 58, wherein detecting interaction of the target protein with the compound comprises measuring the level of enzymatic activity of the PTPase.

59. The method of claim 55, wherein the reaction mixture is a whole cell, and detecting interaction of the target protein with the compound comprises measuring one or more of (i) the level of phosphorylation of a cellular protein, (ii) the level of intracellular Ca^{2+}, (iii) the level of a diacylglyceride, (iv) the level of an inositol phosphate, (v) the level of expression of an immediate early (clE) gene product.

60. The method of claim 55, wherein detecting interaction of the target protein with the compound comprises a determining the amount of the compound bound by the target protein.

61. The method of claim 55, wherein the reaction mixture is a cell-free reaction mixture.

62. The method of claim 55, wherein the reaction mixture is a two hybrid assay.

63. The method of claim 55, wherein the reaction mixture is derived by recombinant expression of the compound in a cell.

64. A method of identifying agents capable of altering the CD19-mediated signal transductioncomprising:

(a) forming a reaction mixture including
  (i) a PTP1C phosphatase,
  (ii) a phosphorylated CD19 protein, and
  (iii) a test agent;
(b) detecting interaction of PTP1C amd CD19,

wherein a statistically significant change in the interaction of PTP1C and CD19 in the presence of the test agent, relative to the level of interaction in the absence of the test agent, indicates an activity of the test agent for altering a signal transduction activity of the CD19 protein.

65. A method to identify compounds capable of modulating SH2 mediated activation of a phosphatidylinositol kinase, the method comprising:

(a) forming a reaction mixture including kinase subunit of a phosphatidylinositol kinase, a polypeptide comprising the acid sequences of CD19 which are capable of binding the kinase subunit

(b) detecting interaction of the kinase and CD19 peptide,

wherein a statistically significant change in the interaction of the kinase subunit and CD19 peptide in the presence of the test agent, relative to the level of interaction in the absence of the test agent, indicates an activity of the test agent for modulating SH2 mediated activation of a phosphatidylinositol kinase.

66. A kit for identifying compounds capable of regulating hematopoietic cell function comprising a PTP1C phosphatase, an ITIM mimetic capable of binding and activating the PTP1C, a PTP1C substrate and a putative regularoty compound.

67. A method for modulating an immune response, comprising contacting a hematopoetic cell with a compound of claim 1.

Figure 1

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	V....V	

Figure 2

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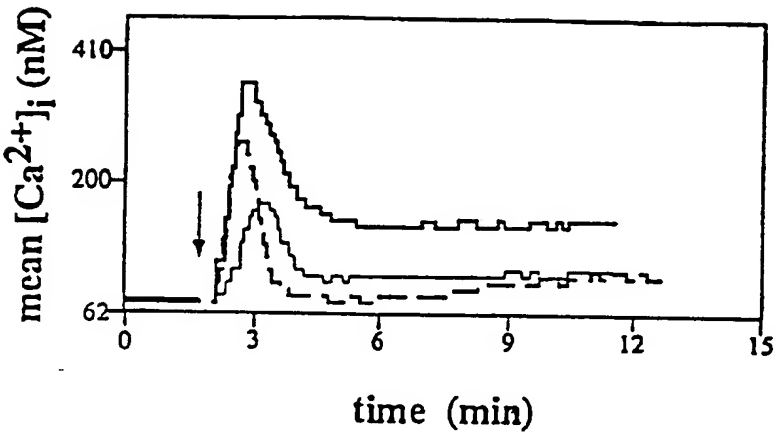
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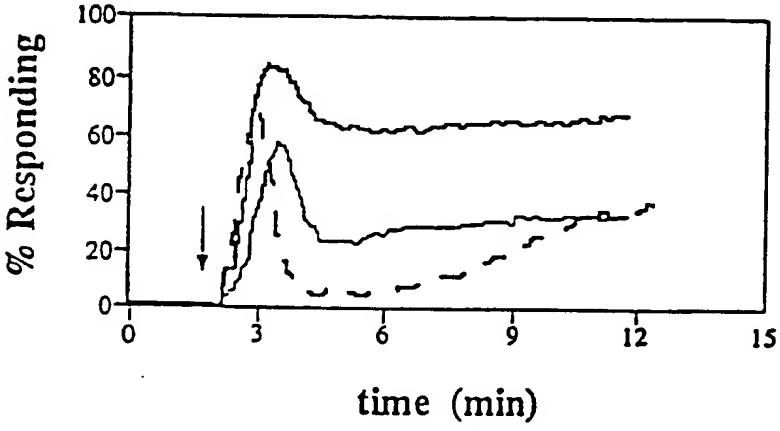
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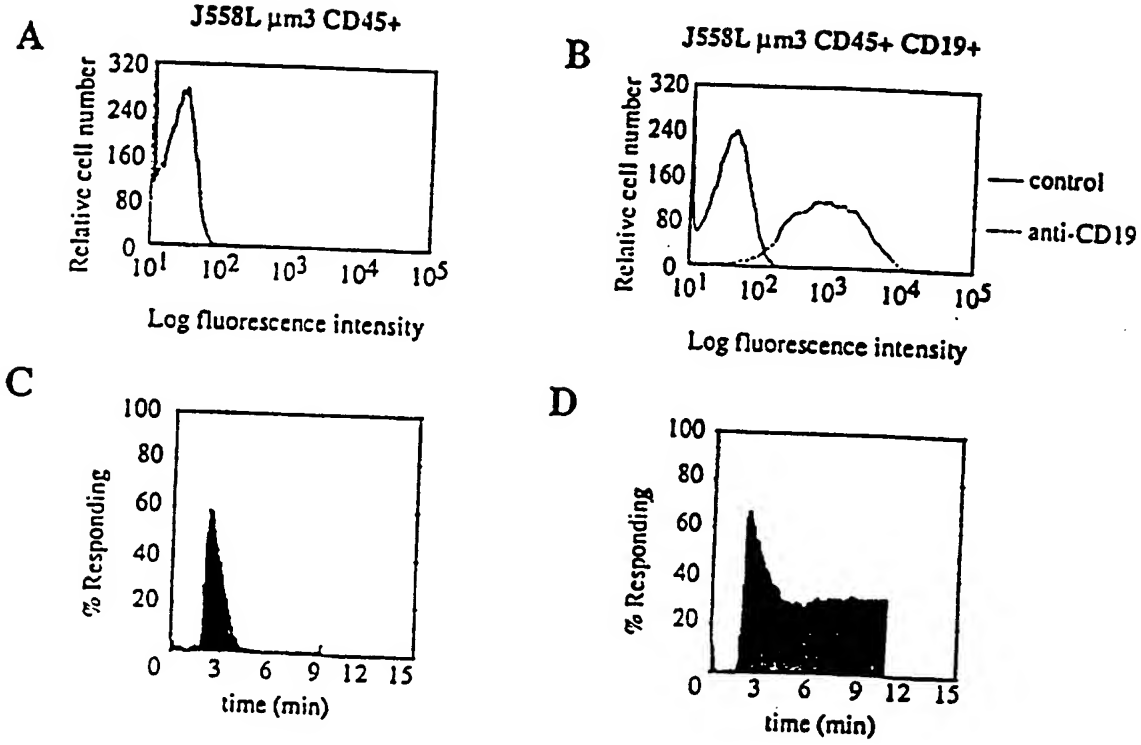
time (min)

Figure 3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Log fluorescence intensity
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240
160
80
0
10^1 10^2 10^3 10^4 10^5
Log fluorescence intensity
control
anti-CD19
C
% Responding
100
80
60
40
20
0
3 6 9 12 15
time (min)
D
% Responding
100
80
60
40
20
0
3 6 9 12 15
time (min)

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(74) Agents: VINCENT, Matthew, P. et al.; Lahive & Cockfield, 60 State Street, Boston, MA 02109 (US).

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(57) Abstract

The present invention is directed to a novel product and method for regulating hematopoietic cell function and a novel product and method for identifying compounds capable of regulating inflammation. The present invention includes a method to regulate hematopoietic cell function by contacting a cell with a regulatory reagent capable of altering the activity of a molecule including PTP1C, PTP1D, ITIM-p160 and ITIM-p70. The present invention also relates regulatory reagents capable of regulating the activity of PTP1C, PTP1D, ITIM-p160 and ITIM-p70, including nucleic acid molecules having sequences that encode such reagents and antibodies raised against such reagents. The present invention also includes a therapeutic composition comprising such reagents and their use to protect animals from inflammation.

p58.183.1	vnredsdeqdpqeVtYaqLnhcvftqrkit--	288-317
p58.183.2	rpsqrpktpptdiIvYteLpnaep--------	318-341
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p58.EB6.2	rpsqrpktpptdiIvYteLpnaesrskvvscp	317-346
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INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/US 96/02289

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IPC 6 C07K14/705 C07K7/02 A61K38/10 A61K38/08 G01N33/53
G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

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IPC 6 C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 25570 A (SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH, USA) 10 November 1994 see the whole document ---	1-4,8, 11-13, 17-67
X	EP 0 432 691 A (YEDA RES & DEV) 19 June 1991 see page 3, line 5 ---	1-4,8, 11-13, 17-67
X	EP 0 304 279 A (UNIV LELAND STANFORD JUNIOR) 22 February 1989 see column 9, line 16 -----	1-4,8, 11-13, 17-67

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 November 1996	11.03.97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer Chakravarty, A

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Information on patent family members

Ir ational Application No
PCT/US 96/02289

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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